













MICRO-DIFFUSION ANALYSIS AND  
VOLUMETRIC ERROR



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AND  
VOLUMETRIC ERROR

BY  
EDWARD J. CONWAY, M.B., D.Sc.

PROFESSOR OF BIOCHEMISTRY, UNIVERSITY COLLEGE, DUBLIN

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TO MY WIFE



## PREFACE

IN the following chapters an account is given of the diffusion principle in analysis, and the applications which have so far been adequately tested in practice both here and in other laboratories. For such methods it is claimed that they save labour, apparatus and time in serial analyses, and can—with some exceptions—be conducted with the accuracy of fine macro work.

In Part I an account is given of the apparatus and principles used. From the latter further applications of the micro-diffusion technique may be deduced for varying conditions and different substances. The description of apparatus includes an account of pipettes, burettes and colorimeters, such as have been used in the working out of the various methods, and throughout the section the question of accuracy with such apparatus has been given prominence. Much of this, it is hoped, may be of aid to the senior student, the chapter on colorimetry being written largely with that intention. The section also includes a description of the 'drop-scale' technique of Dr. Kirk and of Drs. Linderstrøm-Lang and Holter.

Part II contains descriptions of methods with the standard absorption apparatus, and may be directly consulted by those already familiar with the comparatively simple micro-diffusion technique. The reader's attention may be drawn to the special method of suspended absorption used for the blood ammonia determination, since this may be generally applied where errors of the order of a few per cent. are not considered important and makes the method particularly flexible for massed ammonia and other analyses.

In Part III I have attempted to introduce order into the subject of error in micro-volumetric technique, but in doing so have felt it necessary to consider the subject formally both for macro and micro volumes and in particular from the standpoint of the variable error. Some of the material given on the



constant error may be of interest only to the pure chemist, but it is hoped that the chapters on the variable glass and chemical errors will appeal also to the biologist, since the principles expounded govern the choice of micro volumetric technique when working towards expected accuracies.

The case of routine or serial determinations of urea in blood and urine may be considered a special feature of the diffusion technique with the standard absorption apparatus. It is shown also that such methods can be made to give any desirable accuracy in practice. For this reason an Appendix has been included giving an account of the application of such analyses to the measurement of renal functioning.

For their kind permission to reproduce illustrations appearing in their published work, my thanks are due to Dr. P. Kirk, Drs. K. Linderström-Lang and Holter, Dr. D. Richter, V. Stott, and to Dr. D. D. Van Slyke; as also to Messrs. A. Gallenkamp, Messrs. E. Leitz, Messrs. J. Springer, Messrs. G. Thieme, Messrs. H. F. & G. Witherby, and Messrs. C. Zeiss.

I wish to thank as well the editors and publishers of the *Biochemical Journal*, Hoppe-Seyler's *Zeitschrift für Physiologische Chemie*, the *Journal of Biological Chemistry*, the Akademische Verlagsgesellschaft M.B.H. ; and, for the descriptions of certain methods, the authors, as well as the editors and publishers of the Journals mentioned in the text. Finally, my thanks are due to M. Kane of the Architectural School here, for preparing most of the illustrations for reproduction, and to the Irish Medical Research Council for a grant to purchase some of the apparatus described.

EDWARD J. CONWAY

UNIVERSITY COLLEGE  
DUBLIN

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## CHAPTER I

### INTRODUCTORY

IN the development of modern biological research large numbers of observations become increasingly necessary in determining the questions at issue. It becomes essential to have analytical methods of a micro kind in which, without sacrifice of accuracy with respect to macro procedure, the labour and time expended with the single analysis are reduced to a minimum.

In the search for such a method, primarily for ammonia and urea, the micro-diffusion principle of analysis was elaborated here (1, 2) and a special diffusion apparatus and burette (3) designed to give it effect. It was subsequently found applicable to a variety of micro determinations (4, 5, 6, etc.).

The method in general would appear to be the simplest possible consistent with the maximum attainable accuracy in the handling of micro volumes. Distillation and aeration are eliminated, the passage of ammonia or other substance taking place by diffusion from one chamber, in which it exerts a certain tension, into an absorbing fluid in another chamber in which its tension is reduced to zero. With a knowledge of the diffusion conditions controlling this passage and used in the design of the standard diffusion apparatus or Unit, full absorption times with many substances run from about half an hour to two hours.

With serial determinations the time expended on each determination need be only a few minutes; one of the special advantages of the method being the ease with which large numbers of accurate data can be assembled.

With the Unit, the accuracy of ammonia and consequently, of urea and other determinations, is limited only by the accuracy of delivering and titrating fluid volumes of the order of 1 ml. With the development of the titration principles described in the text, this accuracy can be brought to any

C.M.A.



desirable level in practice, so that with comparative ease the percentage error need not exceed that for ordinary macro titrations on the 25 ml. level.

The following is a list of the determinations so far in which the micro-diffusion procedure has been found of value.

- |  |  |
|--|--|
| (a) Ammonia, including special application to normal blood ammonia determinations. | (j) Iodide (in halogen mixtures—not yet described).  |
| (b) Total nitrogen.  | (k) Carbon dioxide—with application to determination of  |
| (c) Urea, with application to renal function tests.                                | (1) blood bicarbonate or 'alkali reserve', (2) oxidation rates of organic substances in vitro. |
| (d) Adenylpyrophosphoric acid.   | (l) Acetone (fine qualitative testing).  |
| (e) Adenylic acid (muscle).  | (m) Alcohol (qualitative and preliminary quantitative).  |
| (f) Adenosine.   |  |
| (g) Aliphatic amines.  |  |
| (h) Chloride.  |  |
| (i) Bromide.   |  |

Besides these determinations, that of micro glucose quantities—using carbon dioxide absorption after yeast fermentation—and nitrates after reduction to ammonia with zinc dust in strong alkali are being at present investigated.

Several of these methods are already in considerable use in other laboratories and have proved very satisfactory. Some have been developed elsewhere; for example, the determination of aliphatic amines or their qualitative recognition by Richter (7) and fine acetone testing by Fearon and Webb (8), as well as the very delicate and accurate total nitrogen determinations by Bentley and Kirk (9).

A special burette was developed (3) with the Unit, the use of which may be also extended to general micro titrations, in particular those of a routine kind where a long permanence of solution and of titre are required combined with ease of refilling and cleaning.

For the technique evolved therefore it may be claimed that it represents a new and special section of micro technique with a fairly wide range of application, and the purpose of the present book is to give an account of the applications which have been tested in practice.

**The general error of volumetric procedure.** During the

development of this technique the more general question of the error involved in volumetric procedure was studied firstly in order to compare the error of the new technique with those already established. Certain principles, however, were in this way discovered of practical help in assigning standards of attainable accuracies, and likely to prove useful in micro work.

To anyone with a laboratory experience it will seem that there are few more practical questions of micro technique than the comparative accuracies in the use of glass ware in volumetric procedure and titration. The constant error of general titrimetric procedure has no doubt been expounded by Bjerrum (10) and others, and the question of exact calibration been long considered. The variable error, however, has not hitherto been equally appreciated, and for the biological worker with his comparative series it is the more important; not only this, but a correct understanding of the variable error affects in turn the question of calibration, and forms much of the rationale of micro volumetric procedure. This latter turns on an equation representing the addition of the variable glass and the chemical errors, which serves also as a means whereby, for given quantities, we can choose ideal volumes.

In the study of the variable error the exact influence of certain factors on the constant error was also discovered, which may be of interest to laboratory workers in general, applying a volumetric technique.

**Note on the description of micro-concentrations.** As is well known the description of the concentrations of standard solutions is generally made in terms of normality or molality (with the symbols *N* or *M* associated with the descriptive number). The immediate results of analysis, however, are more usually stated in terms of the absolute units of mass and volume, such as grammes per ml., per 100 ml. or per litre.

Macro-concentrations may in this way be considered as those most conveniently expressed as grammes per 100 ml. or per cent., and such description may be retained no matter how low the quantity analysed provided the relation of quantity to volume remains on the same level, though for the actual designation of the minute quantities or volumes themselves we have

the milligramme (mg.), and the gamma ( $\gamma$  or 0.001 mg.), also termed the microgramme ( $\mu g$ ) and the lambda ( $\lambda$  or 0.001 ml.—introduced by Kirk, ref. 11).

Micro-concentrations may be expressed as milli-equivalents or as millimols per litre, but analytical results are more immediately given as milligrammes per litre (or per cent.), parts per million, or gamma per ml.

These three units of concentration are alike and the term 'gammil' as the equivalent of the three is suggested by the author as a convenience in the laboratory.

The distinction between the micro-quantity (or volume) and the micro-concentration is in any case one worth drawing, because of its significance in relation to the theory of titration and the variable chemical error (as expounded in Part III of the present volume). From a consideration of the latter the analytical practice with small quantities should be to avoid or raise the micro-concentration by scaling down on the micro-volume. There is a 'gammil' level, however, above which there is little or no practical value in increasing the concentration (taking certain standard precautions of acidimetric micro-titration), and this for ammonia is approximately 10 'gammils', though at 1 'gammil' the error need not exceed 2%.

For colorimetry or spectrophotometry of the ordinary kind 0.3 'gammil' is the order of limit for accurate working (within 2%). This concentration of ammonia nitrogen after Nesslerisation and viewed through a stratum depth of 50 mm. gives an extinction value (E) of approximately 0.3, with filter S 43 of the Pulfrich photometer. An extinction of 0.3 is towards the lower end of the extinction scale for accurate observation.

In comparing such 'gammil' limits, the relation of the molecular weights must of course be borne in mind when these diverge greatly.

**Scale and accuracy of the micro-diffusion methods described.** Using the standard Unit or absorption apparatus, accuracies of the order of 0.5% are easily obtainable for ammonia, urea, chloride, bromide and carbon dioxide at the 20, 50, 250, 500 and 500 'gammil' level respectively. Accuracies of the order of 1-2% can be obtained with one-tenth these

amounts, and of 5% with as low as 0.4 'gammil' of ammonia and a few 'gammils' of chloride and bromide.

With 1 ml. volumes used in the standard Unit, the 'gammil' represents the 'gamma' as the quantity analysed. By scaling down the dimensions of the apparatus and the volumes used much lower quantities could be dealt with having the same concentrations and with the same accuracies. It is in short the 'gammil' level (i.e. the concentration) that sets the possible accuracy and not the quantity or volume considered separately.

The present volume is divided into three parts. Part I deals with the apparatus and principles used in micro-diffusion analysis. Part II gives an account of the methods for laboratory usage. In Part III is expounded a new treatment of volumetric error with particular reference to micro-quantities and the rationale of micro-volumetric technique.

In an Appendix to the volume an account is given of the best as well as the easiest method of applying blood and urine urea determinations to obtain a measure of renal function. This is expressed as a urea '*normality ratio*'. It is shown to be superior to the 'clearance' test and is not directly related to any theory of the mechanism of renal excretion.

The student desiring a rapid account of the essential micro-diffusion technique will find this in Chapters II, V and the first part of Chapter VI, describing the horizontal burette; he will find in Part II a detailed practical account of the various methods.

Numbers referring to the bibliography at the end are printed in italics.



*PART I*

APPARATUS AND PRINCIPLES USED IN  
MICRO-DIFFUSION ANALYSIS

---

*CHAPTER II*

A STANDARD MICRO-DIFFUSION APPARATUS  
OR UNIT

THE standard micro-absorption apparatus or Unit (*I*) consists of a pyrex glass container resembling a small Petri dish with thick glass walls, within which a second chamber is formed by a circular wall of glass arising from the floor. The wall of the inner chamber is about half the height of the outer.

The apparatus is covered during determinations by a square glass lid on which is smeared a small amount of fixative, such as vaselin or a mixture of vaselin and paraffin wax, and in this way makes an air-tight compartment.

Figs. 1 and 1*a* illustrate the standard pyrex unit at present available from Messrs. A. Gallenkamp, Finsbury Square, London.

**Principle of use.** The general principle of the Unit methods consists in the absorption by simple gaseous diffusion of the volatile substance from the outer chamber where it exerts a certain tension into the inner chamber where its tension is zero on the surface of the absorbing fluid. Though this on a first consideration would seem a very slow process, yet from the manner of construction of the Unit the rate is comparatively fast, and determinations may be carried out in about 30 minutes to two hours for a number of different substances. Where large numbers are being carried out the procedure can be so arranged that the average working time for the single determination is only a few minutes. Ammonia analyses by simple gaseous diffusion were carried out by Schlösing (*12*) and by Shaffer (*13*)

over thirty years ago. Separate vessels were used, both being covered by a large bell jar, and upwards of 48 hours with the Schlösing method and 3 to 4 days with the Shaffer modification were required for the absorption, which even then gave some-

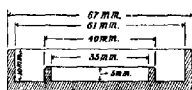
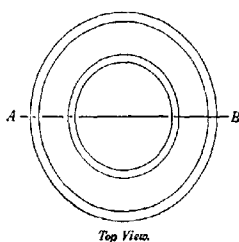


FIG. 1. Plan of standard Unit (from the *Biochemical Journal*, 27, 420).



FIG. 1a. Standard Unit (as supplied by Messrs. A. Gallenkamp & Co., Finsbury Square, London).

what low results. The essential controlling principles of the method were in short not understood and led to its disuse. With the micro-diffusion principle as rationally elaborated here full absorptions can be obtained within the hour.

**Procedure as exemplified by ammonia determinations.** 1 ml. of standard acid is introduced into the central chamber. This may be introduced either from an Ostwald or from a

straight tube pipette, with a delivery time not less than about five seconds, or what is often convenient for routine work, from a 2 c.c. standard burette of the Bang type (14) (*vide* Fig. 6) with comparatively large reservoir. Into the outer chamber is run a measured volume of the fluid to be investigated—usually ranging from about 0.1 to 2.0 c.c. For this also an Ostwald pipette or straight tube pipette may be used. The lid is suitably smeared with the fixative (vaselin, vaselin plus paraffin or a mixture of paraffins) and placed in position on the Unit. The apparatus is then slightly tilted—most conveniently by resting one edge on a spare lid. The tilting collects the fluid in the outer chamber away from where the alkali is introduced. The lid is horizontally displaced so that a small opening appears into the outer chamber sufficient to allow the introduction of the tip of a pipette containing a measured quantity of reagent liberating the volatile substance, which for ammonia determinations is usually one ml. of saturated potassium carbonate.

This is quickly run in *without blowing* and—as its delivery does not require to be very accurate—it is most conveniently run in from a simple pipette made from a piece of glass tubing somewhat drawn out and with a mark at the required volume. The lid is at once replaced and the mixture in the outer chamber rotated about 10–20 times. This is easily done by gripping the Unit between thumb and fingers and tilting slightly from side to side. It will be found that—at least with very little experience—there is no danger of spilling the contents of the chambers, when the volumes described in the determinations are used.

After a suitable time for the absorption the lid is detached and the contents of the inner chamber titrated. For other determinations it may be necessary to remove it for colorimetric investigation.

Variations in the above procedure occur in the different determinations described in text, particularly in the manner of introducing the reagents into the outer chamber. Instead of one such, several may be required, the last reagent liberating the volatile substance.

**Cleaning the Units.** The success of the methods—particularly for the minute ammonia determinations—very largely



depends on a satisfactory cleaning. This cleaning is quite easy, but requires the carrying out of a definite routine.

The following routine of cleaning is found suitable for all determinations.

1. The Units after use are well washed out under the cold water and then under the hot water tap.

2. They are then cleaned of any grease with a test-tube brush or pledget of cotton wool, using a little soap, and subsequently well rinsed under tap.

3. After this they are filled with dilute sulphuric acid ( $N/200$  roughly) containing some of the Tashiro indicator. This acid may be made up in bulk in a large distilled water bottle and filled into each Unit from the outlet of the bottle. They are allowed to soak in the acid for fifteen minutes.

4. They are now well rinsed under the cold-water tap and finally with distilled water. The drops are shaken out (the interior of the apparatus intended for immediate use should never be dried with a cloth) and the Units are left to dry. For quick drying it will often be found very convenient to range them along the top of a radiator, resting upside down along the ridges. They also dry comparatively quickly after shaking out the drops well by mere exposure to the air.

**Important points for correct usage in the general procedure.**

(a) The Units when used for determinations where the final measurement is acidimetric should be perfectly clean in an acid-alkali sense and a routine similar to that given must be followed in cleaning.

(b) The surface of the inner chamber must be fully covered. With the use of a little soap in the cleaning process, as mentioned above, 1 ml. fluid will very easily cover the surface. The introduction of soap means at the same time the introduction of alkali, and hence the reason for soaking in acid for fifteen minutes.

(c) The fluid volume in the outer chamber should not exceed that described in the various determinations. (The fluid volume in this chamber, as shown in the next chapter, has in general a very marked effect on the absorption rate, the time for a 99.5 clearance going inversely as this volume.)

## CHAPTER III

### FACTORS INFLUENCING THE ABSORPTION RATE FROM OUTER TO INNER CHAMBER, WITH SPECIAL REFERENCE TO AMMONIA

IN the practical descriptions of the various methods in their standard form, these factors need not be further considered, but if the conditions are in any way altered, or if new methods are tried out, it is essential to note their general effects.

**The effect of time on the absorption rate.** The rate at which the volatile substance comes across is directly proportional to the tension it exerts in the outer chamber. For ammonia with a comparatively slow absorption rate the tension in the fluid in outer chamber after time '  $t$  ' will be proportional to  $(a - x)$ , where  $a$  is the original amount in the outer chamber, and  $x$  the quantity that has diffused therefrom after time '  $t$  '.

Writing this relation in the form

$$\frac{dx}{dt} = 2.3A_1(a - x), \quad (1)$$

where  $2.3A_1$  is the absorption rate of ammonia per unit quantity in the outer chamber, we obtain on integrating

$$\frac{1}{t} \ln \frac{a}{a-x} = 2.3A_1 \quad (2)$$

$$\text{or } \frac{1}{t} \log \frac{a}{a-x} = A_1. \quad (3)$$

Provided the absorption is not too rapid under the conditions this equation describes approximately the time-absorption curve of ammonia or other volatile substance when this is liberated all at once in the outer chamber. A more general treatment is given in the subsequent chapter.

Fig. 2 shows the time-absorption curve of ammonia at room temperature when to 1 ml. of ammonium chloride solution in the outer chamber is added 1 ml. of  $N/10$  NaOH (Curve A,

22° C.) or 1 ml. of saturated potassium carbonate (Curve *B*, 22° C.).

Curve *A* is described fairly closely by equation 3. Curve *C* shows the rate at which ammonia is absorbed from 0.5 ml. ammonium chloride solution plus 0.5 ml. saturated potassium carbonate, and Curve *D* the absorption rate at 38° C. from 1 ml. solution plus 1 ml. carbonate.

In the chloride determinations the total amount of chlorine finally absorbed is not liberated at once in the outer chamber.

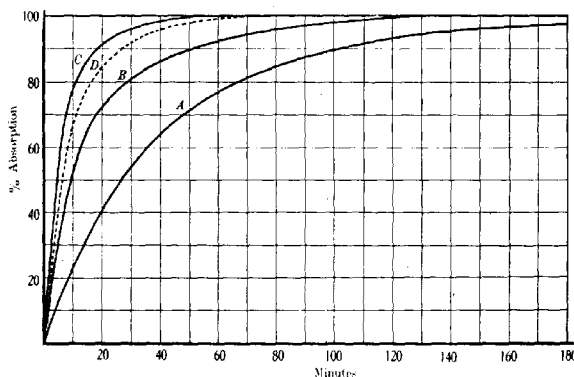


FIG. 2. Ammonia absorption curves in standard Unit.

Inner chamber containing 1 ml. *N*/200 sulphuric acid, outer chamber containing for

<i>A.</i>	1 ml. <i>N</i> /150 am. chlor. plus 1 ml. <i>N</i> /10 NaOH.	Temp. 23.
<i>B.</i>	" " " " " " sat. $K_2CO_3$ .	" "
<i>C.</i>	0.5 ml. " " " " 0.5 ml. " "	" "
<i>D.</i>	1 ml. <i>N</i> /150 am. chlor. " 1 ml. " "	" 38.

The oxidation reaction proceeds rather slowly, and the resulting curve of absorption is a straight line up to an absorption of about 70%. (See Fig. 34.)

**Effect of the fluid volumes on the absorption rate.** The fluid volume in the inner chamber has practically no influence on the absorption rate provided the surface is fully covered. This is due to the fact that it operates by reducing the tension of the absorbed substance to zero, and therefore provided it is in excess, a change of volume has no significant effect. It is essential in the use of the Unit that the surface of the inner

chamber should be fully covered. The Unit is so designed that 1 ml. of fluid readily covers the surface and is in general the most suitable volume to use, though for the most delicate determinations 0.7 ml. will be found sufficient.

The fluid volume ( $V$ ) in the outer chamber has by contrast a very important effect on the absorption rate, which—other things being equal—is inversely proportional to this volume. This arises from the absorption rate being directly proportional to the tension, and hence to the concentration of the volatile substance in this chamber. If a certain quantity of substance is dissolved in 1 ml. fluid it will exert a tension twice as great as when dissolved in 2 ml., the fluid having otherwise the same composition. By comparison the absorption will require twice the length of time in the latter case to clear the substance to the extent of 99.5% from the outer chamber. Mathematically we may express the relation (similar to equation 1) as

$$\frac{dx}{dt} = A_2 \frac{a-x}{V}, \quad (4)$$

where  $A_2$  is the absorption rate per unit concentration ;  $2.3A_1$  as in equation (1) being the rate per unit quantity.

$$\text{From (4)} \quad \frac{V}{t} \ln \frac{a-x}{a} = A_2 \quad (5)$$

$$\text{or from (3)} \quad A_2 = 2.3A_1V. \quad (6)$$

The relation of absorption rate of fixed quantity to changing volume is shown in Table I.

TABLE I

Quantity of ammonia in outer chamber (millimols).	$V$ = volume of fluid in outer chamber ml.	Time, allowed for absorption mins.	$A_1$ value = $\left(\frac{1}{t} \log \frac{a}{a-x}\right)$ .	$A_2$ value = $2.30A_1V$ .
1.0	1.0	16	0.0153	0.0352
1.0	2.0	26	0.0080	0.0368
1.0	3.0	24	0.0056	0.0386
1.0	4.0	22	0.0041	0.0377

Temperature = 18.5°.

Two estimations were carried out for each volume. The  $A_2$  value represents the calculated absorption rate in millimols per minute per unit concentration of the ammonia from the outer chamber, unit concentration being here 1 millimol per ml.

There are certain qualifying conditions for the simple inverse relation of fluid volume to absorption rate, but it should be borne in mind as an underlying relation. It is necessary, for example, that the surface of the outer chamber be fully covered; secondly, in practice the fluid in this chamber is generally altered in composition as the total volume alters, and this can affect the relationship; finally, if the rate of formation of the volatile substance is markedly slower than its absorption rate (as with chlorine from chloride under the standard conditions) the simple inverse relation does not hold.

**Effect of the saline content of mixture in the outer chamber.**

The effects of the saline content of the outer chamber are given in Chap. X for ammonia absorptions and the results would appear applicable also to the aliphatic amines, which are briefly considered subsequent to the ammonia determinations.

**The effect of temperature.** All the determinations described may be carried out at room temperature. At the same time the absorption rate can be hastened by placing the Unit in an incubator, and this is suitable for ammonia determinations in general, and doubtless for other absorptions, though with the fixative in present use for the halogens and the most delicate ammonia determinations it is inadvisable, owing to the fluidity of this fixative at the raised temperature.

Incubating at 38 degrees reduces the necessary absorption time for ammonia determinations to about 60-70% of that at room temperature. This will be again considered when dealing with ammonia determinations.

**The effect on the absorption rate of altering the dimensions of the apparatus.** The standard apparatus at present procurable from the makers is suitable for the various determinations described in Part II. For special purposes, however, alterations in the dimensions may be required, in which case the effect of such alterations will be useful to know. Where the clearance distance between the inner and outer chamber or the distance of the inner chamber from the lid roof is not greatly altered, the following principles apply approximately.

(a) The absorption rate is proportional to the geometric mean of the surface of the inner and outer chambers.

(b) It is inversely proportional to the mean distance of passage of the molecules.

For the application of principle (a) the two surfaces must be fully covered.

From the operation of the first of the two principles it will be seen that the absorption rate will be directly proportional to any change in the general linear dimensions of the standard apparatus. An apparatus designed with all dimensions doubled will give twice the absorption rate, and if halved will give half the absorption rate. The utilisation of this to increase the rate of absorption is limited by the fact that as the linear dimensions increase the volume required to cover the surface increases as the square of these dimensions.

As shown above, the time of 99.5% clearance of the volatile substance is proportional to the volume in the outer chamber. It will be seen that if we keep the volume in the outer chamber in all cases just what covers this surface, we will get the fastest relative absorptions or the shortest times for clearance of the substance as we *decrease* the linear dimensions.

TABLE II

Area of floor of outer chamber cm. <sup>2</sup>	Area of floor of inner chamber cm. <sup>2</sup>	Height of inner chamber cm.	Height of outer chamber cm.	Distance of molecular passage * cm.	Absorption rate found ( $A_2$ value - $2.30A_1 \times V$ )	$A_2$ divided by the geom. mean of the areas multiplied by the distance of passage of mols.
66.8	43.5	1.0	2.0	13.9	0.091	0.023
22.7	16.6	0.6	1.6	8.6	0.064	0.028
43.1	14.8	0.8	1.3	9.6	0.062	0.024
7.9	17.8	0.5	1.0	7.0	0.047	0.027
16.7	9.6	0.5	1.0	6.9	0.044	0.024
25.6	8.6	0.85	1.65	8.3	0.037	0.021
8.5	1.76	0.80	1.40	4.4	0.025	0.028

Temperature = 23°.

\* The mean distance of the molecular passage from the outer to inner chamber would be very difficult to state, but it has been taken as proportional to the total diameter *plus* the height of the outer chamber *minus* the fluid depths in the two chambers (the total diameter of the outer chamber is taken at the top and thus includes that of the inner chamber).

The absorption rates have been determined with pure ammonia solutions (N) in the outer chambers and N sulphuric acid in the inner. The figures in

Halving these dimensions will then halve approximately the time required for ammonia absorptions.

The best utilisation of these principles will depend on the special analytical conditions. The standard apparatus is designed with the most suitable general dimensions, in which 1 ml. covers the central chamber without any trouble and in which the outer chamber surface is also conveniently covered by 1 ml. of fluid plus 1 ml. reagent. Table II summarises the results obtained with a number of units of different dimensions to the standard apparatus.

**Effect of rocking on the absorption rate.** An increase in rate of absorption may be obtained by a simple rocking device with motor—Fig. 3—the Units (*D*) resting upon a hinged platform (*C*) supported by an excentric wheel (*A*) making contact underneath the platform with glass (such as a glass slide fixed underneath).

It is a curious fact—which is explained by the considerations in the following chapter—that where the conditions already exist for a fast absorption (use of saturated carbonate in outer chamber with ammonia or the absorption of substances like carbon dioxide, etc.), the rocking increases this markedly, but when the absorption is already slow there is little effect.

The rocking device converts an absorption rate of ammonia of 0.030 ( $A_1$ ) to 0.045 at room temperature, or will change the full absorption time of  $1\frac{1}{2}$ –2 hours to about 1 hour (1 ml. ammonia solution in outer chamber plus 1 ml. saturated carbonate); a much lesser proportionate effect is obtained when  $N/10$  sodium hydroxide is used to liberate the ammonia. Increasing the period of rocking beyond a certain point (about 60 per minute) has no further action on the absorption rate.

the last column show a fair constancy and are indicative of the general principles underlying the effect on the absorption rate of changes in the dimensions of the apparatus. Divergences from the mean value of these figures are largely attributable to irregular differences in the depth of the air passage between the inner and outer chambers.

It may be pointed out that the linear dimensions of the first and fifth type of Unit investigated above are very nearly in the ratio of 2 to 1, and it will be observed that the absorption rates in the last column but one are also nearly in the same ratio, for these Units.

The absorption rates given in the last column but one are the  $A_2$  values representing the rate of absorption per minute per unit concentration in the outer chamber, or  $2.30A_1 \times V$ .

*Rocking is not advised except for special purposes, since it will obviously tend to diminish the working ease and simplicity of the method.*

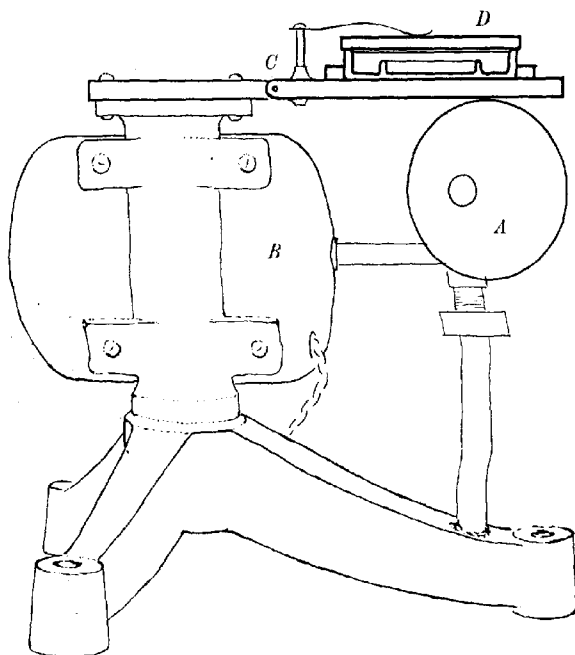


FIG. 3. Rocking device for units if required for special experiments.  
With the general use of the Unit it is not required.

**Other factors.** The time absorption relations are more fully treated in the subsequent chapter, and special factors will be referred to when dealing with particular determinations.



## CHAPTER IV

### GENERAL PRINCIPLES GOVERNING THE ABSORPTION TIME IN MICRO-DIFFUSION ANALYSIS

In the previous chapter an account was given of the practical effects on the ammonia absorption of varying the more important factors. This also applies very largely to the absorption of other substances, particularly those like ammonia, which are not only volatile but very soluble.

In the present chapter the general principles governing the absorption rate of any substance are theoretically derived and are summarised in simple formulae.

The full solution of the general problem of the successive and continuous diffusion of a substance through three media, the conditions in each of which are different, is complicated and scarcely lends itself to useful application in analytical procedure. It will be seen, however, that the practical requirements are sufficiently met by a limitation to special cases.

#### **I. Time required for 99.5% clearance of a volatile substance from a solution stratum of 'a' cm. depth when the tension at the upper surface is maintained at zero.**

The solution of this problem is contained in the equation,

$$\frac{100-y}{100} = \frac{8}{\pi^2} \left[ e^{-\frac{k\pi^2 t}{4a^2}} + \frac{1}{9} e^{-\frac{9k\pi^2 t}{4a^2}} + \frac{1}{25} e^{-\frac{25k\pi^2 t}{4a^2}} + \dots \right], \quad (7)$$

where 'y' is the percentage saturation remaining, 'k' the diffusion coefficient and 't' the time. When y is 99.5% and k of the order of  $1 \times 10^{-3}$  (cm.<sup>2</sup>/min.) for ammonia and other molecules of a similar magnitude through water, this series is very rapidly convergent and only the first term need be considered. From this,

$$0.005 = 0.81 \times e^{-\frac{k\pi^2 t}{4a^2}}. \quad (8)$$

If we consider ammonia with a diffusion coefficient of  $1.09 \times 10^{-3}$

at  $20^\circ$  and  $1.66 \times 10^{-3}$  at  $38^\circ$  (from Landolt Bornstein's Tables and Hufner's data in same tables reckoning for  $20^\circ$  and  $38^\circ$ ) we obtain

$$t = 2.05 \frac{a^2}{k}$$

$$= 1880a^2 \text{ for ammonia at } 20^\circ$$

$$\text{or } 1230a^2 \text{ ,, ,, ,, } 38^\circ \quad (9)$$

Where 'a' is 1 mm. (corresponding to 1.7 ml. in outer chamber of Unit)  $t = 19$  minutes at  $20^\circ$  C. and 12 minutes at  $38^\circ$  C.

For a depth of 1.2 mm. (2 ml. in outer chamber)  $t = 27$  minutes or about half an hour at room temperature and about 18 minutes at  $38^\circ$  C.

When the layer depth is 5 mm. it would require 307 minutes or five hours for a 99.5% clearance at  $38^\circ$  C., and this, quite independently of the volume of fluid used. *For micro-diffusion analysis the stratum depth below the emission surface is therefore of the greatest importance.*

## II. Time required to reach a steady state when a gas diffuses from a fixed tension at one end of a cylinder to zero tension at the other.

In a similar way it may be shown that the time for a steady state is given by the relation

$$t = \frac{2l^2}{\pi k}, \quad (10)$$

where 'l' is the cylinder length and 'k' the gaseous diffusion coefficient. The diffusion coefficient of oxygen into air is  $10.7$  ( $\text{cm.}^2/\text{min.}$ ) as found by Obermayer (15) at zero centigrade. On the basis that the diffusion coefficient is inversely proportional to the square root of the molecular weight and that the temperature effect is given by  $k = k_0 \left( \frac{T}{273} \right)^n$  where  $n$  is 2 (Loschmidt 16; Stefan 17) we may deduce the gaseous diffusion coefficient of ammonia as 16.9 at  $20^\circ$  and 19.1 at  $38^\circ$ .

With the mean distance of passage in a Unit of about 3 cm., the time required is only 0.34 minute at  $20^\circ$  and 0.30 at  $38^\circ$ . It is obvious therefore that only a negligible time is required for reaching a steady state or for the attainment of gradients

which are simply proportional to  $\frac{p}{l}$ , where 'p' is the tension in outer chamber and 'l' the mean distance of molecular passage.

### III. The time required for passage of a volatile substance into an absorbing medium.

This we may regard as a variant of *I*, and consider that a minimal value will be given if we consider the time required for a certain percentage clearance of the absorptive material itself, when one side is maintained at zero concentration.

When acid is being neutralised and the residual acid subsequently determined, then the time required for about an 80% neutralisation will be only the fraction of a minute, a layer of 1 mm. depth being considered, it being noted also that the diffusion coefficient of hydrochloric acid through water is over twice that of ammonia.

From the foregoing, it is clear that the only appreciable delay in the total absorption in apparatus of the standard type, occurs from the diffusion through the liquid layer in the outer chamber, and the time required to unload the quantity of volatile substance across a gradient expressed by  $p/l$ . The times for the attainment of uniform gradients across the gas layer and the time of absorption in the central chamber are comparatively negligible.

It will also be seen that no matter how high the tension of the volatile substance is, and no matter what arrangement we may make with regard to the disposition of the surfaces, the total absorption time from layers of 1.2 mm. depths (2 ml. in outer chamber of Unit) and without agitation of the apparatus, will take about half an hour at room temperature. We could, however, work with lesser volumes in the outer chamber and still cover the surface, though this becomes difficult with 1 ml. We could also, if necessary, much decrease the absorption time by rocking.

**Factors entering into long absorption times.** Where a large amount of the volatile substance produces a relatively small tension the absorption time will be comparatively long. Ammonia will serve as an example. At 38° with 1 ml. of

$N/20$  ammonium chloride plus 1 ml. of  $N/10$  NaOH in outer chamber of Unit, the total absorption time (99.5%) is about two to two and a half hours. Here the diffusion in the liquid layer of about 1 mm. depth and the time for steady gradients in the gaseous layer are comparatively unimportant.

The following factors mostly determine the absorption. The rate of the absorption at any time from the beginning of the absorption will be determined by the gradient expressed as  $p/l$ , or, if diffused quantities are to be calculated, then more conveniently as  $\frac{c_a}{l}$ , where  $c_a$  is the concentration of the gas as g./ml. in the air layer in immediate contact with the outer chamber or diffusing surface, '  $l$  ' being the mean distance of passage. The absorption rate will be also proportional to the area of diffusing surface, or when the diffusing and absorbing surfaces are different in extent to  $\sqrt{s_t s_a}$ , where  $s_t$  and  $s_a$  are the area of the diffusing and absorbing surfaces respectively (shown experimentally as described in preceding chapter). We have then

$$-\frac{dq}{dt} = \frac{c_a}{l} \times \sqrt{s_t s_a} \times k, \quad (11)$$

where  $k$  is the diffusion coefficient of the gas or volatile substance in air. We can express  $c_a$  in terms of the solubility as

$$q = c_a \times S \times v, \quad (12)$$

where  $S$  is the solubility as g./ml. produced by 1 g./ml. in the gas layer, and  $v$  is the volume in the outer chamber and of the quantity dissolved therein.

$$\text{Then} \quad -\frac{dq}{dt} = \frac{q}{S \times v \times l} \times \sqrt{s_t s_a} k. \quad (13)$$

Integrating, we get

$$2.3 \log \frac{q_0}{q_0 - q_t} = \frac{t}{Svl} \times \sqrt{s_t s_a} \times k, \quad (14)$$

and where 99.5% have been absorbed from the outer chamber

$$2.3 \times 2.3 = \frac{t}{Svl} \times \sqrt{s_t s_a} \times k$$

or

$$l = \frac{5.3Svl}{\sqrt{s_t s_a} \times k}. \quad (15)$$

This equation shows the theoretical proportionality of time to volume in outer chamber and to length of mean distance between the surface as shown experimentally in the previous chapter.

From this equation we may make a calculation of the absorption time for ammonia in the Unit at 38 degrees, when 1 ml. of ammonium solution is alkalinised with 1 ml. of  $N/10$  NaOH.

We have

$$V = 2.0 \text{ ml.}$$

$l = 3.8$  cm. (as an approximate figure for the mean distance of molecular passage, being the total distance proceeding vertically from the middle of outer chamber to half the distance from top of inner chamber to roof, across and down at right angles touching a diameter of the floor of inner chamber at one-half the distance from edge).

$S =$  the solubility (g./ml. per g./ml. in gaseous layer). The solubility of ammonia is very much greater at low tensions than at high, which latter are usually listed in solubility tables, the gas not obeying Henry's Law at high pressures. For  $N/1$  ammonia Abegg and Riesenfeld (18) found a tension of 13.45 mm. at 25 degrees. The figure would be slightly increased by the presence of  $N/20$  NaOH—which increase may be neglected. At 38 degrees, assuming the temperature effect to be similar to that at high pressures, we would get a tension of 21.8 mm. In  $N/1$  ammonia we have then 0.017 g./ml. produced by or producing a tension of 21.8 mm. or  $\frac{M}{22000} \times \frac{21.8}{760}$  in g./ml.

The solubility ( $S$  value) is therefore  $\frac{0.017}{2.2 \times 10^{-3}}$  or  $0.77 \times 10^3$ .

$k = 19.1$  (cm.<sup>2</sup>/min.) at 38°, as given above.

Inserting these values in the equation (15), we get

$$\begin{aligned} t &= \frac{5.3 \times 0.77 \times 10^3 \times 2 \times 3.8}{12.7 \times 19.1} \\ &= \frac{31.0 \times 10^3}{242} \\ &= 128 \text{ minutes.} \end{aligned}$$

This corresponds well with the time found experimentally, which, as already stated, is somewhat over 120 minutes.

If now, instead of ammonia, we consider a gas such as chlorine, the solubility at  $38^\circ$  is 0.48 g./100 ml. for 760 mm. tension. We can convert this to solubility in the above units by multiplying by  $\frac{2.2}{M} \times 10^2$ , the  $S$  value being then 1.49 as contrasted with 770 for ammonia.

The  $k$  value for chlorine at  $38^\circ$  may be reckoned, from that of ammonia above, as 9.4.

Inserting these chlorine values in equation 15, we get an absorption time of about 0.5 mins. For chlorine and a number of such substances of comparatively low solubility when compared with ammonia, therefore, practically the whole time of the absorption will be taken for the diffusion clearance from the fluid layer in the outer chamber.

For ammonia with 2 ml. in the outer chamber and a coefficient of  $1.09 \times 10^{-3}$  at room temperature, this is 27 minutes or just half an hour. Keeping the same volume relations, this will be the order of figure for a number of substances since the diffusion coefficient will go as the square root of the molecular weight.

Thus chlorine and bromine from chlorine and bromine water, and carbon dioxide from acidified bicarbonate are found experimentally to be fully or almost fully absorbed after 30 minutes, 40 minutes and about 30 minutes respectively, the diffusion proceeding from 2 ml. volumes. Iodine goes in a similar relation from iodine water or oxidised iodide, being somewhat but not much slower, as may be expected.

**Summary of the factors influencing the absorption times in micro-diffusion analysis at constant temperature, and in particular for the standard Unit.** (1) In micro-diffusion vessels with distance between emission and absorption surfaces of the order of 3.5 centimetres or less, and where the solubility of the substance at 760 mm. pressure is about 0.5 g./100 ml. or less (this being multiplied by  $\frac{2.2}{M} \times 10^2$  for use in the present treatment), the time for a 99.5% absorption is given by

$$t = 2.05 \frac{a^2}{k},$$

where 'a' is the depth in centimetres and 'k' the diffusion coefficient through water (cm<sup>2</sup>/min.).

For 2 ml. fluid in the outer chamber of the Unit 'a' is 0.12 cm. and the absorption time for a number of substances will then lie between 30 minutes and 1 hour.

The time absorption curve for such substances will follow a diffusion curve and not the logarithmic relation given in the previous chapter.

(2) Where the solubility is high (upwards of 50 g./100 ml. or more in the Units given above), and where the gaseous tension of the volatile substance is low for a standard concentration, the absorption times will be comparatively slow, and the liquid diffusion (with 1 mm. layer) comparatively unimportant. This is so for ammonia when the fluid in the outer chamber is 2 ml. and the tension has not been raised by potassium carbonate or other substance. The time in minutes for a 99.5% absorption is then given by the general relation,

$$t = \frac{5.3Sv}{\sqrt{s_l s_a} \times k},$$

where *S* is the solubility in g./ml. for a gaseous concentration of 1 g./ml. (This is  $\frac{2.2}{M} \times 10^3$  times the usual figure given as g./100 ml./760 mm. pressure.) *s<sub>l</sub>* and *s<sub>a</sub>* are the surfaces of emission and absorption in (cm.<sup>2</sup>); *v* is the volume in the outer chamber (ml.); 'l' is the mean distance of passage of molecules, and 'k' the diffusion coefficient (cm.<sup>2</sup>/min.) of the gas or volatile substance through air. With 2 ml. in the outer chamber this equation for the standard Unit reduces to

$$t = 3.2 \times \frac{S}{k}.$$

(Solubilities should here be reckoned for low tensions, since Henry's Law may not be obeyed at high pressures.)

The time absorption curve in this case follows a logarithmic relation of the kind given in Chapter II.

(3) Rocking if used will have a much greater effect on the rapid absorptions than on the slow type, since in the former

the time of absorption is almost altogether due to the diffusion gradients through the liquid which are easily broken up by rocking. With the slow type of absorption due to high solubility, rocking has very little effect, for the uniform diffusion gradient through the gas layer will be scarcely disturbed, and the liquid diffusion gradients are comparatively unimportant.



## CHAPTER V

### PIPETTES (SUITABLE FOR USE WITH THE STANDARD UNIT) AND THEIR DELIVERY ERRORS

HERE it is not proposed to give a general account of pipettes, but only of such as have been used in practice in the various determinations, subsequently described with the Unit.

For delivering fluid volumes of the order of 2.0 to 0.1 ml. the following may be considered.

- (a) Simple pyrex tube pipettes, of length about 15 to 20 cms.
- (b) Ostwald pipettes, or pipettes of Ostwald type.
- (c) Standard pipettes similar in form to the general standard macro-pipette.
- (d) Pipettes delivering between two marks.
- (e) The standard 2 ml. Bang burette.

**Deliveries into the central chamber.** The volumes required for the central chamber deliveries range from 0.7 to 2.0 ml. Where accuracies of the order of 0.1 to 0.2% (standard deviation) only are required, any of the above may be used, the pyrex tube pipette delivering in not less than 4 seconds. Where accuracies of a higher order are needed (down to 0.02%) the best is the simple tube pipette, and the worst is probably the pipette delivering between marks (d), since the rate of this delivery is usually irregular.

**The simple tube pipette.** (To deliver volumes ranging from 0.7 to 2.0 ml., Fig. 4, *E*.) These are easily made from pyrex tubing with internal diameter of about 3.0 mm. for the 1 ml. delivery. A section of tubing is drawn out in the flame and cut to a length of about 30 cm. The tip should be fine as in Fig. 4, *E*, and the time of delivery of about 20 cm. length with tube held upright being noted. If the accuracies of 0.1 to 0.2% are required it should not take less than 3 seconds to deliver, the delivery time being regulated by constriction in the flame. 1 ml. is now sucked up (this 1 ml. being delivered by an Ostwald or 2 ml.

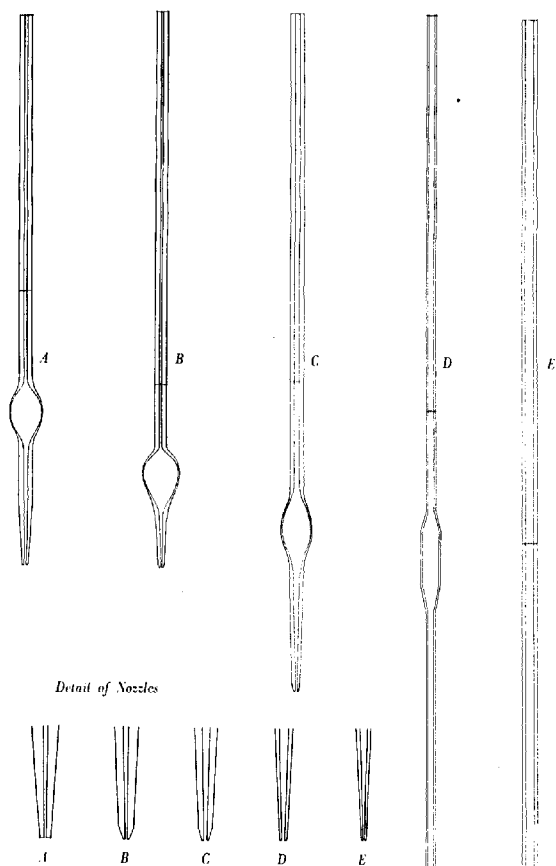


FIG. 4. Drawings from actual samples. The Ostwald pipettes *A*, *B* and *C*, are representative samples now sold, in each case inferior to the original, by reason of deliveries too fast, or of too much glass to orifice at tip (*A*), or of ends too sharply bevelled (*B*, *C*). The simple tube pipette *E*, taking 40 seconds to deliver, is much superior in accuracy to *A*, *B*, *C* or *D* (*D* is from a standard 1 ml. pipette).

burette into a small tube) and a level a little above the meniscus marked with a little gummed paper. The pipette is then standardised for delivery. Whatever volume is delivered is divided by the length of the pipette, and in this way any small difference in level of mark to bring to 1 ml. exactly is noted, and a mark made at this point with a small sharp file. The pipette is then finally standardised.

TABLE III

<i>No. of pipette.</i>	<i>Kind of pipette.</i>	<i>Volume ml.</i>	<i>Delivery time. Secs.</i>	<i>Variable error as coeff. of variation.</i>	<i>Manner of using.</i>
1.	Straight tube, fine tip	0.73	40	0.029	Drop blown out gently after delivery, rotating tip against side.
1.	Same straight tube, some tip removed	0.73	4.8	0.096	As in previous.
2.	Ostwald	1.0	2.2	0.12	As in previous.
3.	Straight tube	0.96	2.5	0.13	As in previous.
4.	Straight tube	1.0	1.2	0.16	15 seconds drainage with tip touching side.
5.	Straight tube, wide tip	1.0	0.1	0.40	Drop blown out sharply after delivery with tip against side.
5.	Straight tube, wide tip	1.0	0.1	0.41	Tip touching glass floor after delivery, drop blown out sharply.
1.	Straight tube, more of tip removed	0.73	0.1	0.48	Drop blown out sharply after delivery, tip against side.
5.	Straight tube, wide tip	1.0	0.1	0.76	Tip touching fluid for 2 seconds after delivery.

The various straight tube pipettes had lengths from 13-18 cm. from mark to tip.

From Table III it is obvious that by far the most important factor in accuracy is time of delivery. The coefficient of variation with the first pipette listed—0.029—is practically the same

as that found with a 25 ml. standard pipette. The pipette was a simple tube pipette with fine tip made in the laboratory in a few minutes. The total time taken for its delivery was not more than 41 seconds, that of the 25 ml. pipette—45 seconds, including drainage. From this it would not be surprising if a 1 ml. technique came to supersede entirely the 25 ml. macro technique.

For reading the meniscus a carefully made file mark will suffice, but an etched ring may also be made by first coating with melted paraffin, rotating as this cools, ringing around with a fine metal point or knife edge and then painting with hydrofluoric acid.

A background formed by a small piece of white label gummed behind the mark will be found suitable.

Elaborate attempts to secure increased accuracy with the meniscus reading are largely waste of time, particularly if the main factors influencing the pipette error are not appreciated.

With regard to the accuracy of delivery, we may consider briefly what exact meaning is to be attached to accuracy in such matters. In pipetting 1 ml. by any such means we deliver what is a perfectly definite amount of fluid, which can be weighed and its exact volume ascertained. When, however, we come to make a second delivery under the same conditions, it is a fact that this may deviate from the first, and if we were to assemble a large number of such observations, it would be found that plotting as ordinates the number of times a given deviation from the mean occurred, against the plus or minus deviations, we would obtain a more or less regular curve according to the number obtained. This curve (Fig. 41, Part III) would be symmetrical around the mean and tail down rapidly on each side. It may also be constructed if we know the standard deviation from the mean, and the practical limits of the curve are included in plus or minus three times the standard deviation. Consequently the standard deviation, or alternatively the probable error (0.67 time the standard deviation), gives us the required measure of accuracy.

The standard deviation as a percentage of the mean (coefficient of variation) is

cient of variation) is given for a simple tube pipette—as shown in Part III—by the relation

$$V_p = \frac{0.022}{\sqrt{dt}},$$

where ‘ $d$ ’ is the internal diameter of the tube in cm. and ‘ $t$ ’ the mean time in seconds for the meniscus to fall 1 cm.

For reaching fine accuracies with any such pipette it is essential that the tip be fine, with a small proportion of glass surface to orifice at tip, and a fine taper with no sharply shelving shoulders. Fig. 4, *E* illustrates the kind of tip desirable, which is a little finer than that in use with the standard 1 ml. pipette and much finer than that for many Ostwald pipettes sold commercially.

Immediately after the delivery, the drop in the tip, which should touch the glass, is gently blown out, the pipette being rotated.

Results from pipettes of such a kind and also of a coarser type of pipette are given in Table III. It appears from this table that our error will decrease from 0.48% to 0.029% (standard deviation) merely by altering the time of delivery; and that the error found with a 0.73 ml. simple tube pipette delivering in 40 seconds is practically as low as the error with the standard 25 ml. pipette. This delivers with a coefficient of variation of 0.025%, taking altogether about 45 seconds when drainage is included.

*It is obvious however that time and care will often be economised by considering the required accuracy with reference to the maximum unavoidable error in the whole analysis.*

**The Ostwald pipette.** The original Ostwald pipette, introduced over forty years ago, had the form shown in Fig. 5, which is taken from the 2nd edition of the *Physiko-Chemischer Messungen*, by Ostwald and Luther (19) (1st edition in 1893). This original Ostwald pipette is still the best of its kind, subsequent variations or attempted improvements merely diminishing its accuracy. The following is a translation of its description: “The delivery end is made from the same glass tube as the body of the pipette, the neck which is about 1 mm.

wide, being joined on. It is essential that the delivery end should be narrow, passing down in a slightly conical manner with walls not too thin; the tip is narrowed in the flame—considerably increasing the durability—and so regulated that the free delivery of the contents takes  $\frac{3}{4}$  minute. With such pipettes the procedure previously described with blow out and with the tip touching the wall is essential for exact measurements.”

In many subsequent representations of the Ostwald pipette the original delivery time is considerably diminished (as in pipettes from which *A*, *B*, and *C* of Fig. 4 are drawn), thereby removing its essential feature; one also meets with inconvenient relations of bulb to delivery tube (Fig. 4, *B*), and of too great a proportion of glass to orifice at the tip (Fig. 4, *A*). One may find also that the bulb is provided with rather sharp angles tending to collect air bubbles.

The accuracy of the original Ostwald pipette depends very largely on its slow delivery and fine delivery tip tapering without any sharp bevelling at the end; but the fact remains that a simple tube pipette delivering in the same length of time gives an equal accuracy. The lesser internal surface of the Ostwald pipette would seem at first to be an advantage, but the great variation in rate of fall of meniscus acts against this advantage, and in any case a slight extra increase of delivery time in the single tube would compensate.

It may be held that the 1 mm. bore of the neck gives a lesser reading error than the 3 mm. bore of the simple tube. This question of the reading error is here of negligible importance. With tubes of such a bore and slow delivery, it is very easy to arrange the meniscus sharply at the mark. The semicircular meniscus is clearly demarcated and a deviation from the mark



FIG. 5. Original form of Ostwald pipette, from Ostwald-Luther's *Physiko-Chemische Messungen*. Leipzig, 1902.

of 0.1 mm. is as great as need ever be present, and with experience it should be less. A possible deviation of the meniscus from the mark of 0.1 mm. in a 1 ml. tube of 20 cm. length means a standard deviation of 0.014%. This in adding to 0.03% will increase it only to 0.034% (since they add as the square root of the sum of the squares).

A further advantage of the straight tube pipette is that the residual fluid can be always calculated if necessary, and in accordance with the formula (as shown in Part III) :

$$F = 3.45dl \sqrt{\frac{\bar{d}}{t}},$$

where  $F$  is the wall fluid in c.mm.,  $l$  the length in cm, and  $d$  and  $t$  have the same significance as in the previous formula.

**The 2 ml. standard Bang burette and the graduated pipette.** Both of these are alike in that a delivery of the order of 1 ml. is made from one mark to another, but the burette is more convenient for routine fillings, since the meniscus is controlled by a tap and not by the finger, and filling from the reservoir makes it also agreeable to work with. Neither is superior to the simple 1 ml. tube.

Fig. 6 illustrates a convenient form of the 2 ml. Bang burette. (See next chapter for its description.)

**Deliveries into the outer chamber.** For delivering into the outer chamber the same list of pipettes may be considered, the 2 ml. burette being excluded. The range of delivery is anything from 0.1 ml. or less to 3.0 ml. In delivering into the central chamber the use of one fixed pipette or burette for one type of determination is obviously the most suitable. Here the question arises as to the advisability of having a series of pipettes for the single delivery. It would seem best on the whole to use here also the one pipette standardised for delivery volume. This may be washed out directly after each delivery and then water, alcohol, ether and air sucked through after attaching to the filter pump. In this way it is rapidly cleaned and dried. Alternatively, the pipette may be washed out with small volumes of the next fluid prior to its filling to the mark and subsequent delivery of the fluid into the outer chamber.

After such deliveries subsequent washing may be carried out when only 0.1 or 0.2 ml. volumes are used. It must not be

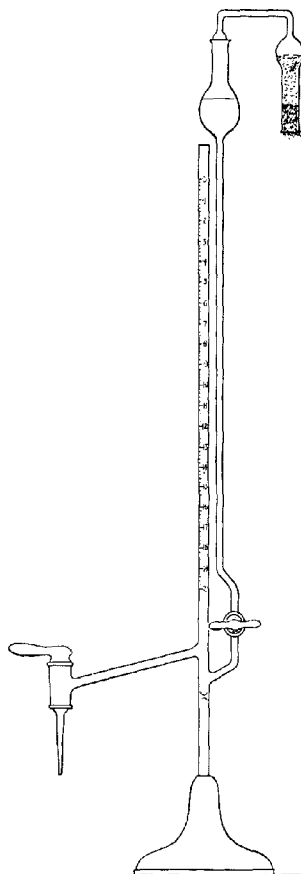


FIG. 6. 2 ml. Bang burette, with soda-lime tube attachment.

forgotten that increasing the volume of the outer chamber increases the time for full absorption, and unless this is allowed for, serious errors can arise.



For a discussion of the effects of drainage time, blowing out of drop, etc., the reader is referred to Part III. Here it may be said that no increased error is introduced when the drop is blown out immediately at the end of delivery—which can be sharply enough determined by watching the meniscus—the point of the pipette being rotated against the glass wall at the instant of delivery.

**Standardisation.** In standardising any of the above pipettes five deliveries are weighed out into a tared weighing bottle and the mean weight calculated. The temperature of the water is noted and the volume of the delivery for 20° C. is obtained by

$$V = W \left( 1 + \frac{\gamma}{100} \right),$$

where  $\gamma$  is a correction found in Table XXXII, Part III,  $W$  the weight of water, and  $V$  the standardised volume.

**Cleaning of pipettes.** If pipettes used with the present technique be cleaned with concentrated sulphuric acid and bichromate, they must be very thoroughly rinsed. Sucking through successively water, alcohol, ether and air will generally be sufficient. This is not readily possible with the 2 ml. Bang burette, which may be treated as exceptional and cleaned with sulphuric acid and bichromate. It is essential after full washing out of the sulphuric acid, that the taps be taken out, well washed, dried and re-greased. The fluid for use should also be allowed to remain 24 hours in the burette, which is then emptied and refilled.

When acid of strength less than  $N/500$  is used no exception should be made to excluding concentrated sulphuric acid for cleaning purposes.

**Storage bottles.** With each horizontal burette there is a pyrex storage bottle which holds sufficient fluid for a large number of refills. Otherwise where weak standard acid is being stored, pyrex bottles or paraffin-lined bottles should be used when the acid is of less strength than  $N/100$ . Bottles may be lined by paraffin by firstly drying well, then heating under the hot water tap and pouring in some melted paraffin (M.P. over 50) with which the internal surface is easily covered, subsequently pouring out excess and allowing to cool in air.

Barium hydroxide solutions—as required for deliveries into the central chamber in carbon dioxide determinations—or other weak standard alkali should be stored in a bottle with soda lime guard and siphon delivery. The barium hydroxide may be delivered then directly into a reservoir of a 2 ml. burette or into a small tube with subsequent removal of 1 ml. by a long pyrex pipette, the tip of which is inserted to the bottom of the tube.

Effective prevention of the entrance of carbon dioxide from the air can be made either by painting rubber stoppers, etc., with melted paraffin or by tying lightly a paper collar around the neck of the bottle and using it as a support for melted paraffin poured in. On setting an effective seal is formed.

## CHAPTER VI

### MICRO-BURETTES (SUITABLE FOR USE WITH THE STANDARD UNIT) AND ERRORS INVOLVED IN THEIR USE

In titrations where we are restricted to minute quantities, it is necessary to keep the final volume of fluid low. In this way we decrease the amount of added reagent necessary to produce an observable change around the end point.

The accurate delivery of small volumes needs special burettes. With the Bang 2 ml. standard burette the percentage variable error in delivering 1 ml. is usually (but not necessarily) greater than the percentage error in delivering 25 ml. with the ordinary 50 ml. burette. That it is not necessarily greater will appear from the considerations in Part III.

With a sample 2 ml. Bang burette of standard type the variable error in delivering 1 ml. was found to be 0.11% as a coefficient of variation. The variable error in delivering 25 ml. from a standard 50 ml. burette is of the order of 0.05% (including the reading errors in meniscus adjustment). Such errors are determined for the one burette under the same conditions as standard deviations of the single delivery from the mean of a large number. They are conveniently expressed then as percentages of the mean or coefficients of variation.

Owing to the large reduction of volume and provided we are dealing with small quantities of acid (less than  $10^{-2}$  milli-equivalents) increased accuracy over the 50 ml. would be secured with the 2 ml. Bang burette, the advantage being greater the smaller the quantity. At the same time the use of solutions ten times or so more concentrated is an extra advantage.

With micro-titration in general it is occasionally required to titrate quantities so minute that a burette delivering volumes of the order of 0.1 ml. or less is necessary, and such a burette is very suitable for Unit determinations. If the principle of

delivery from a vertical burette is retained, adequate control is difficult and drainage errors are likely to become prominent. Such difficulties are removed by delivering from a horizontally placed graduated tube with uniform delivery rate secured by a small hydrostatic pressure.

The error can be made at least as small as with Rehberg's burette (20), (in which the fluid column is expelled by a mercury column controlled by a screw), but the ease of construction and cleaning is greater.

For such a horizontal micro-burette the variable error in delivering 0.1 ml. (the delivery time taking about ten seconds) was found to be 0.12% or practically the same as that found with the 2 ml. Bang burette delivering 1 ml. Such delivery errors—as discussed in Part III—are very dependent, however, on the time required for the delivery, but in so far as the two burettes examined represented two actual burettes in use and typical of their kind, it will be seen that not only is the horizontal burette more suitable for the titration of small quantities, but may be substituted for the Bang burette over the whole range of micro-titration.

The horizontal burette of present dimensions, as described below, permits the use of fluids ten times more concentrated than with the 2 ml. Bang burette and under better conditions of security from contamination by the carbon dioxide of the air or by alkali from glass surfaces, the delivered fluid having been in contact only with pyrex glass walls.

In the author's experience the horizontal micro-burette of design described below is the most suitable for routine work with the standard Unit. The 2 ml. standard Bang burette is also useful, and no doubt various other types will be found serviceable for the particular conditions, including the Rehberg burette, which has proved of so much value in micro-work and can be readily adapted. These three burettes are described in the ensuing section.

(a) **The horizontal micro-burette (3).** A suitable design of the burette for general purposes is shown in Fig. 7 (and may be procured from Messrs. A. Gallenkamp, Finsbury Square, London).

*A* is a piece of thermometer tubing graduated in 0.01 ml. and 0.001 ml. large and small divisions. Being of good quality thermometer tubing uniformity of bore is secured. It is joined by a small piece of rubber pressure tubing to tube *B*. To *B* is attached the delivery tube and tap *C* and also the connecting tube and tap *D*, communicating with the container *E* on a platform which can be raised or lowered from the side and fastened in position by a screw. Behind tap *D* is a small window in the wooden supporting frame to allow free manipulation. All tubing except the graduated thermometer tubing is of pyrex glass. The tube adjoining the graduated tube has

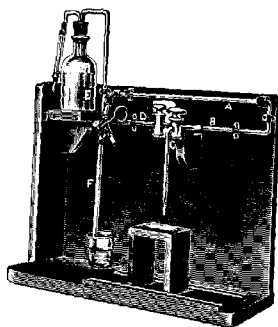


FIG. 7. Horizontal microburette (as supplied by Messrs. A. Gallenkamp & Co., Finsbury Square, London).

twice the internal bore of this latter, so that the volume of the fluid in the graduated tube is comparatively small and the emerging volume has been in contact only with pyrex glass.

The cleansing required for a smoothly running meniscus is very easily done by detaching the graduated tube and cleaning by any of the ordinary methods. It may as a rule be cleaned sufficiently well using alcohol and ether. After cleaning it is replaced with the glass ends in contact and washed out with a few drops of fluid from the container. The small hydrostatic pressure ensures a uniform delivery without drainage owing to the horizontal position. If the rate of delivery is found inconveniently rapid a simple and efficient method of adequately controlling this tendency consists in attaching a glass tube to

the end of the graduated section as in Fig. 7 (*F*). This dips into water below, contained in—say—a 25 ml. beaker. By sucking up some water in this tube and attaching the clip as in Fig. 7, the delivery pressure of the burette is diminished and controlled as desired. The internal diameter of tube (*F*) should be at least 4 mm. The delivery tube should at the same time be finely pointed and not too blunt and the opening sufficiently constricted to give a comparatively slow flow.

In delivering from the burette the tip of the delivery tube may be allowed to touch the fluid for titration. If this method is not used a fine pyrex rod will be found suitable for removing small volumes of fluid from the tip and an amount representing one division on the graduated tube may be controlled in its emergence and transferred to the titrated fluid. Much finer deliveries could be secured by the contact method.

*Filling, greasing of taps, etc.* The burette is firstly assembled in accordance with the diagram, and the various parts compared with the description. The manner of usage can be readily deduced from the construction, but the following description may prove helpful. The tube *F* is detached from tube *A* and the sliding platform for the Unit also removed. The pyrex bottle *E* is detached, its supporting platform being lowered, after turning the screw at the side. The bottle *E* is rinsed out with some of the fluid for use, then filled to near the top and replaced in position. It will be necessary at first to suck the fluid across into *A* by attaching a thin rubber tube to the end of *A* and then sucking. The rubber tube is detached and the fluid allowed to drip from *A*, the overflow being caught in a vessel placed suitably underneath. The delivery tap at *C* is opened and the fluid allowed to flow out freely, being, if necessary, gently sucked out at first through a rubber tube attached to the delivery tip. When all the air has been displaced, the taps are turned into the closed position, the sliding platform for holding the Unit is replaced and some titrations carried out to see if the apparatus is in working order. The tube *F* may then be attached and water sucked up if it is thought desirable to have a better control of the meniscus in *A*. When filled and in working order the burette requires no attention for a long

period, other than the tube *A* being occasionally detached for cleaning when necessary with alcohol and ether for a smoothly running meniscus.

If at any time the taps begin to stick, they are opened after detaching bottle *E*, dried with filter paper, as also their glass sockets after emptying the tubes, then greased in the usual manner and replaced. In greasing such taps a very small quantity of rubber grease is smeared on and then a minute amount of vaseline, which is spread by rotating the tap in socket.

Where extremely weak barium hydroxide solutions are used (of the order of  $N/1000$ ), each day before the series of titrations about 10 ml. of the alkali is run out and discarded; all rubber connections—cork of reservoir bottle and rubber of soda-lime guard—should likewise be sealed from the air with melted paraffin, and their surface should be as small as is conveniently possible.

(b) **The Bang burette (14).** The most suitable burette of this type is the standard 2 ml. with soda-lime guard as shown in Fig. 6. The fluid from the bulb-like reservoir is controlled by the tap below and behind the graduated section. In filling this latter bubbles are generally easily removed by having the tap fully open at first. With a well-made delivery tip the fall of the meniscus down to the 1 ml. level should not take less than ten seconds.

After some time in use the meniscus will not descend evenly and droplets remain on the sides, when the burette must be cleaned. Strong sulphuric acid and dichromate may be used, the burette being filled with this and allowed to stand overnight. It must then be repeatedly rinsed out with water and subsequently with the fluid for titration, being allowed to stand several hours with this and refilled before titrating.

The remarks concerning the suitable greasing of taps in the previous section apply here also, and before using, the new burette should be cleaned and the taps freshly greased.

*The end-point emergence from burettes.* Before using the horizontal micro-burette for titrations the minimal or end-point emergence for careful routine work should be observed. For

the 50 ml. burette this is usually taken as the drop; for all burettes below this volume the drop is too large and, of course, far too large for the 2 ml. Bang burette and the horizontal burette. With a well-made Bang burette the end-point emergence should not exceed 4 to 5 c.mm. For the present horizontal burette it need not exceed 0.5 c.mm. Such an emergence is transferable with a fine pyrex rod from the tip of the burette delivery tube to the fluid for titration. Finer emergences than this are secured by the contact method of delivery, which has now become rather general in micro work (21). Larger emergences—1.0 or 2.0 c.mm.—may be used when the titration does not call for the finest accuracy.

On turning the tap of a burette carefully until a small amount of fluid emerges it will be noticed that the tiny emergent gush is rather constant for each single burette. It depends on the degree of constriction and the glass surface at the end of the tip, as well as on the volume and shape of hole bored through the movable tap and the extent to which this is greased, minimal greasing giving the best control.

Where in the case of the horizontal burette it is found difficult to control delicately the end-point emergence it will be found of assistance to diminish the hydrostatic pressure by sucking water up in tube *F* (Fig. 7) until the emerging rate with fully opened tap is going rather slowly. A marked tendency of the meniscus to jump several divisions may be removed by carefully regreasing the taps with the least amount of material. If this does not remove the excessive jumping the delivery tip of the burette should be still further constricted.

*Precision of reading meniscus.* It is of practically no advantage to read the meniscus to less than half the end-point emergence, but it is of advantage in the most accurate work to read it at least so far. This will mean the reading of the 50 ml. and 2 ml. Bang and horizontal burettes to 0.2 of a division. Most frequently it will be necessary to read them only to half or one division.

(c) *The Rehberg burette.* In the Rehberg burette the titrating solution is on the top of a mercury column controlled at the bottom by a screw, which is mercury tight. Pincussen's



modification (22) of this burette is shown in Fig. 8. In this model a greater ease of filling is secured by the insertion of the two-way tap. With this the fluid in the graduated section is connected with the delivery capillary or with the filling reservoir.

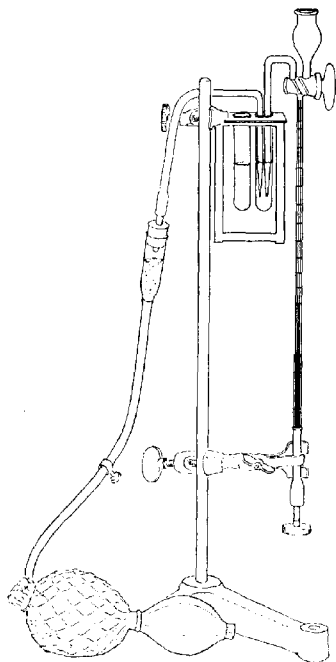


FIG. 8. The Rehberg burette, from Pincussen's *Mikromethodik* (Thieme, Leipzig), showing attachment for bubbling through alkali.

The graduated section consists of a thick-walled capillary tube of 0.1 ml. capacity between the terminal graduations, of which there are altogether 100. The reading of the mercury may be easily made to 0.25 of a c.mm. by means of a lens. The delivery tube bent twice at right angles and somewhat finely drawn out is of similar bore to the graduated section, with which it may be connected by suitably turning the tap. Below the

graduated section the tube is widened and fits into the screw and plunger arrangement, to which it may be securely fixed by metal spirals as in diagram. Before using the screw is turned so that the plunger is well down when the lower section is filled with mercury and attached securely to the upper part.

The burette is fixed to a stand which also carries an adjustable holder for two small test-tubes backed by a window of milk glass. The apparatus is so arranged that the delivery tube of the burette enters one of these tubes carrying the fluid to be titrated and passes down almost to the end of this. The other tube is used with similar fluid volumes and indicator and buffered to the exact end-point required.

The stirring arrangement as shown in Fig. 8 is carried out by bubbling air through the titration fluid, the air pressure being established by a rubber pump such as is used in ether freezing and controlled by a screw clip. For acidimetric work the air is passed through a soda-lime tube and then through a glass tube bent at right angles and ending rather finely at the tip.

*Preparation for titration.* After fixing the two parts of the burette, with the plunger low and the metal spirals in position, the two-way tap is turned so that the graduated section connects with the reservoir. The mercury column is raised by twisting the screw until it just appears in the reservoir, which is then filled with the titrating fluid. The mercury is now lowered and the fluid drawn into the graduated section, after which the tap is adjusted, so that the fluid in burette connects with the delivery section and this is filled. The tap is turned again to connect with reservoir and the mercury lowered to a little below the zero reading. The reservoir is then shut off and a little of the fluid is sent out through the delivery tube on carefully turning the screw until the mercury stands at the zero point. Any adhering drop is removed from the tip of the delivery tube by touching with filter paper. The test-tube containing the solution for titration with indicator and companion tube are then placed in position as in Fig. 8, the air current cautiously started and the titration begun.

*The use of the Rehberg burette with the standard Unit.* At the

end of the absorption period the fluid in the central chamber is pipetted into the titration test-tube, followed by two washings of distilled water of about 0.5 ml. each, the fluid with washings being then titrated.

**The three burettes compared.** The principles of accuracy in the use of burettes is formally considered in Part III, and below, certain details are given concerning the accuracies of the horizontal and Bang burettes in routine working. Here, however, the leading features of the three may be briefly considered. Such burettes must be judged both from the point of convenience and accuracy. It is obvious that two burettes of equal working accuracy are not of equal value if one requires the more attention in use. Of the three burettes it would seem that the horizontal burette is the most convenient for routine work. Compared with the Bang or Rehberg burettes it has relatively a much greater reservoir capacity and a greater ease of reading—which can be carried out from the sitting position—in large numbers of serial titrations. It is much easier to clean effectively than the Bang burette—since only the detachable graduated section need be cleaned, and this may be effectively done with alcohol and ether. Its greater essential simplicity than the Rehberg burette is an advantage ultimately in convenience, to which may be added the consideration that it works independently of the contained fluid, whereas oxidising fluids cannot be readily employed with the mercury and screw principle without sacrifice of some of its advantages.

On the point of accuracy, it is shown in the next chapter that the horizontal burette is somewhat more accurate in use than the 2 ml. standard Bang burette, and particularly so for very weak solutions. Compared with the Rehberg burette, it may be said that the working accuracy of a burette depends on its manipulation and delivery errors. Under the term manipulation error is included the reading error and that resulting from the magnitude of the end-point emergence. It is very likely that the reading error and the delivery error will be similar in both, the delivery error being due to variability of the wall fluid left behind, the mercury sliding over this wall fluid in the Rehberg burette. The delivery error can be

indefinitely diminished as the delivery rate decreases, which is more fully considered in Part III. There remains the question of the end-point emergence. Here it may be held that the mercury with screw gives the finer control. It is the author's experience, however, that any practical degree of control can be reached with the simple horizontal burette, depending on cutting down the hydrostatic pressure by tube *F* and clip, and by increasing the fineness of the delivery tip.

In the following section, a brief account is given of the errors involved in the use of the horizontal and the Bang burette. (The subject is more fully dealt with in Part III.)

**The variable error in titrating 1 ml. of standard sulphuric acid with the horizontal compared with that for the 2 ml. Bang burette.** By variable error is here meant either the standard deviation or the coefficient of variation of the single titration with respect to the mean of a large number (these terms are defined in Chapter XXIII, Part III). The error of pipetting out the 1 ml. acid will not be taken into account, the burette error being alone considered. This latter—as considered more fully in Part III—arises from the pure delivery error and the manipulation error. The latter is in turn a composite error arising from the reading and the end-point errors.

These various sources of error may be increased or diminished to a considerable extent according to the degree of care and time expended on the titration. We shall consider what may be taken as typical results of careful working in which the end-point emergence of the two burettes is 4.0 c.mm. and 0.5 c.mm. respectively. (With badly made burettes such emergences may not be obtainable.)

We shall suppose that the reading is brought to 0.2 of a final division, and that the delivery error of each burette is the same, namely 0.12%, as a coefficient of variation.

The sulphuric acid for titration is taken as similar to that used in the ammonia determinations subsequently described and contains 0.0012% methyl red and 0.0002% methylene blue with 20% alcohol; and that the volumes delivered from each burette are always 2.0 ml. and 0.2 ml. respectively.

From such data the curves in Fig. 9 are constructed and are in accord with the experimental findings, but for a fuller understanding of their nature Part III must be consulted. (The indicator could be used in lesser concentration and 0.0008% methyl red is found suitable for blood ammonia titrations, and even lesser concentrations are workable.)

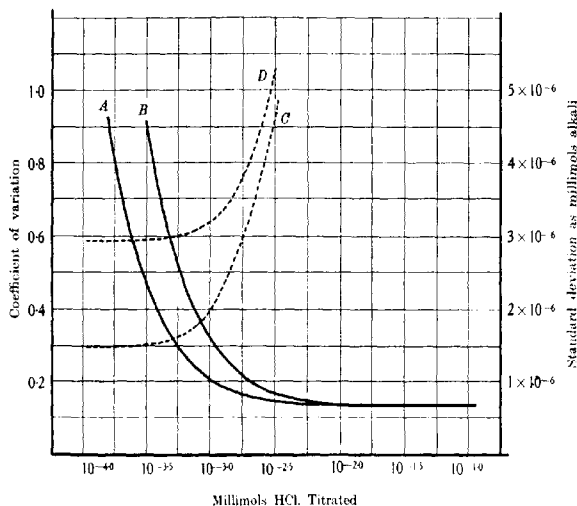


FIG. 9. Curves of variable error in titrating 1 ml. hydrochloric acid with barium hydroxide solution (or carbon dioxide free alkali), using 0.2 ml. from a horizontal burette (Curve A) and 2.0 ml. from a Bang burette (Curve B).

Curves C and D give the corresponding variable error in millimols of alkali (reckoned as KOH).

Two sets of curves are given in the figure, one group (A, B) representing coefficients of variation, and the other (C, D) the variable error in millimols. Each group of curves shows two definite regions and an intermediate section. With A and B, to the right we have pure glass error which when expressed as coefficients of variation (0.14% for each burette) is independent of the quantity of acid titrated until this falls to about  $10^{-2.5}$ , and towards the left we have a region of pure chemical error, which is inversely proportional to the quantity titrated and directly proportional to the end-point volume. This variable chemical

error which dominates at the lowest acid levels for titration, arises from the indefinite nature of the end-point change as the alkaline strength decreases.

**The variable error of titrating 1 ml. of 0.0002N sulphuric acid illustrating the minimum variable error of acidimetric titration.** The object of determining this error is to ascertain the least amount of alkali or acid which can be discriminated in practice by titrating 1 ml. with the burette. If we consider the titration of 1 ml. of  $N/10$  acid delivered, for example, from a 1 ml. simple pyrex pipette (drawn out tube, length 17.5 cm.), the standard deviation with the horizontal burette is usually  $25 \times 10^{-5}$  millimols. Using  $N/100$  fluids the standard deviation in millimols is ten times less, and further reduction in the strength of the fluids will progressively reduce the standard deviation expressed in absolute units. We reach a point, however, when the standard deviation so expressed becomes constant. This limit of standard deviation sets the limit of our discrimination and is proportional to the total buffering power of the fluid at the end-point multiplied by the indiscernible range here of the  $pH$  figure.

The limit for 1 ml. titration is reached for the present horizontal burette when the quantity of acid titrated is in the region of  $10^{-3.5}$  millimol (as shown in Fig. 9).

For titrations in atmospheric air it is not possible to diminish this figure appreciably for the above volume by titrating closer to a  $pH$  of 7.0, for any diminution of the buffering power of the water secured thereby is more than counterbalanced by the rapidly increasing buffering power of the carbon dioxide of the air as well as by the instability of the end-point.

The carbon dioxide of the air will be in equilibrium with distilled water of approximately 5.6  $pH$ , so that the methyl red indicator has the great advantage that its point of maximum sensitivity lies in the same region.

In a carbon dioxide free atmosphere, and titrating 0.7 ml. acid to a  $pH$  of 7.0 with the above glass ware and similar fluids and volumes, it is possible to decrease the standard deviation from  $1.1 \times 10^{-4}$  to about  $0.3 \times 10^{-6}$  millimol KOH, provided we substitute for the indicator mixture one equally sensitive at

a *pH* of 7.0 and provided also our alkali is perfectly carbon dioxide free.

**General uses of the horizontal burette.** Besides its use in determinations with the standard Unit the horizontal burette, as already mentioned, may be employed with advantage in general micro-titrations. It can also be used to give—and that quite easily—accuracies of the same order as when 10.0 to 15.0 ml. are titrated from the 50 ml. burette, with coefficients of variation of 0.16 to 0.11%. Accuracies of a higher order than this can be obtained—as considered in Part III.

The long delivery tube in the present horizontal burette is useful for titrating into centrifuge tubes, small beakers, etc.

It has been used with advantage, for example, in the thio-sulphate titrations of the Hagedorn-Jensen micro-glucose determinations in blood, using *N*/20 thiosulphate instead of *N*/200, having therefore the advantage of greater permanence in the titrating fluid. It has also been used in micro-permanganate titrations of calcium oxalate, and with such, an automatic stirrer may be conveniently set up by fixing a capillary tube connected with an air blower along the delivery tip (though this cannot be done with iodine owing to the iodine volatility).

## CHAPTER VII

### THE MICRO-DIFFUSION METHOD WITH END-POINT VOLUMES AROUND 20 CUBIC MILLIMETRES

THE use of the method for such small end volumes and those of a similar order in the inner chamber, will rarely arise, and only for very special conditions. It is obvious that the standard Unit can be scaled down to any desirable level for use with capillary pipettes and narrower gauge burettes than those already described. With regard to absorption times, from consideration of the principles in Chapter III, and keeping the fluid depths at about 1 mm. and the surfaces of the glass chamber covered, the necessary absorption time on scaling down approaches the limit for clearance from the outer liquid by diffusion. This is 19 minutes at room temperature and about 12 minutes at 38°. Reducing the depth to 0.5 mm., a possible 5 minutes total absorption time could be obtained at room temperature.

The narrower gauge burette on the present horizontal model has not yet been tried in practice, but with the burette as it stands, a perfect control to the smallest fractions of an ultimate division can be obtained by drawing out the delivery tip to a fine point, so that with fully open tap it delivers at the rate of about 30 seconds for 0.1 ml. The titration of 10 c.mm. with 10 c.mm. would require the reduction of the bore of the graduated horizontal tube to about 0.3 mm. and a careful standardisation or certification for bore uniformity. Also the delivery tip will need to be specially fine, and dipping into the titration fluid.

**Kirk's technique.\*** In the method for 'drop scale' volumes (i.e. about 20 c.mm.) a modification of the standard Unit was constructed (Gibbs and Kirk, 23), the linear dimensions being

\* (The description follows closely that of Kirk and of Kirk and Gibbs, in *Mikrochemie*, 11, 24.)





FIG. 10. Kirk's modification of the standard Unit for drop scale volumes, 32 mm. outside diameter (from *Mikrochemie*, 16, p. 26, 1934), *vide text*.

reduced to about one-half (Fig. 10). It may be made from glass tubing of about 32 mm. outside diameter, by making the end a test-tube bottom, sucking this in, and with the use of a fine flame, blowing a central cup. After this the tube is cut and ground on the top, so as to be readily sealed with a small flat glass plate. Besides the diffusion cell a special capillary burette was also constructed and suitable pipettes for measurement of samples, etc.

*Procedure.* The procedure of the analysis follows the general method already described, except that the drops are held compact, spreading being prevented, if necessary by adding traces of vaseline to the wash water in cleaning. For ammonia or urea determinations 0.025 to 0.050 ml. standard acid ( $N/20$  HCl) containing methyl red is introduced into the central chamber. The sample containing ammonia is measured into the outer chamber, subsequent to the introduction of a drop of about 0.1 ml. saturated potassium carbonate, and on the opposite side to this. The cell is then covered tightly with the glass plate greased with either a good stopcock lubricant or with a vaseline-paraffin mixture (vaseline and paraffin in ratio of 3 to 1). Following this the cell is tilted and tapped, causing the merging of the alkali and sample, the mixture being then run around the chamber until thoroughly mixed and the cell incubated 2 hours, after which all the ammonia is absorbed by the acid in the central cup, which is subsequently titrated with  $N/10$  NaOH from the capillary burette, using a magnetic glass stirrer (*vide* subsequent description).

Urea is also determined in a similar manner, introducing 0.550 ml. urease solution (containing phosphate) beside the sample drop and mixing after covering, then incubating at about  $37^{\circ}$  and finally mixing with the alkali drop, etc.

Using this method, Gibbs and Kirk determined ammonia and urea quantities ranging from 1.55 to 8.27  $\gamma N$  in pure solutions and in blood and urine. The mean error involved approxi-

mated to one per cent. They showed, *inter alia*, that small quantities of urea added to blood were quantitatively recoverable, and that urea analysis by the micro-diffusion method gave similar results with whole blood as with Folin-Wu filtrates.

**Kirk's technique of pipetting, stirring and titration.**

*Pipette.* This consists of a simple capillary pipette (11), into the top of which is sealed a fine hypodermic needle, using Kronig cement (24). A 0.5 ml. tuberculin syringe is used to control the fluid measured, and may be used with a series of pipettes of different volumes. After delivery the pipettes are rinsed out with water, thus eliminating drainage error.

*Magnetic stirrer.* A diagram of the stirrer used (slightly modified) is given in Fig. 11. A wooden block carries an electro-

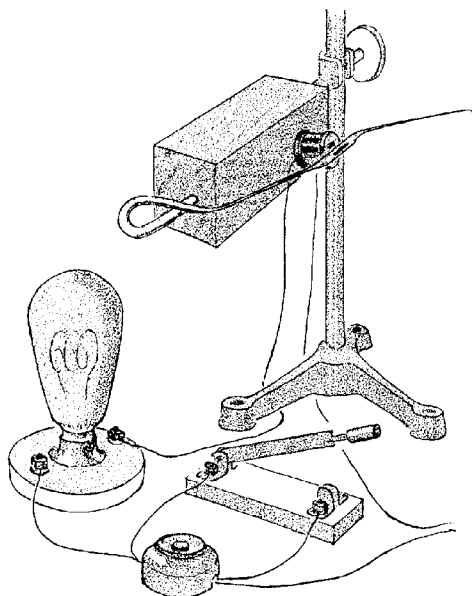


FIG. 11. Magnetic stirrer. Slightly modified from the diagram of Kirk (11).

magnet removed from a small electric buzzer. A glass tube is also inserted in the block and bent so that it passes in front of

the magnet core at about 2 mm. distance. Opposite the magnet core, a piece of iron is sealed inside the tube, above which point the tube is drawn out to obtain flexibility, and also drawn out below this point to terminate in a glass thread which bends down a little at the tip. A 110 watt resistance-lamp is inserted in circuit with the magnet as in diagram, the circuit being closed either by a push button or switch. On application of 110 volt alternating current the stirrer vibrates in correspondence with the frequency of the alternating cycle. This produces a violent local agitation with swirling action in the drop, giving no difficulty from spattering or spreading of liquid. The apparatus is mounted entire on a swivel clamp, so that the glass thread may be applied at any angle to the liquid.

*The burette.* The burette used (Fig. 12) is of the Rehberg type (20), in which the titrating solution is on the top of a

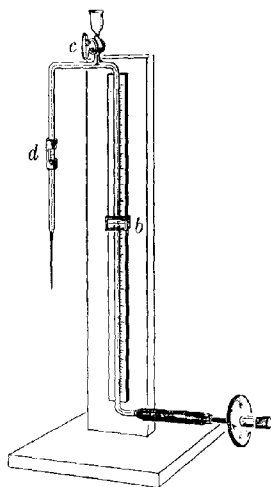


FIG. 12. Modified Rehberg burette as used by Kirk. (From *Mikrochemie*, XIV, p. 6.)

mercury thread controlled at the bottom by a plunger with screw. "The mercury control device is of steel and consists of a plunger 2 mm. in diameter working within a steel cylinder. The plunger bearing is made mercury tight by the use of a metallised wood gasket fastened inside the steel jacket. The plunger is manipulated by means of a screw with 40 threads to the inch and 4 cm. in length. On the screw is mounted a large circular head for fine adjustment and a smaller knurled head for rapid rotation. This entire device slips over the glass tube, being fastened to it by means of de Khotinsky cement. The

upright measuring tube (of thick-walled capillary tubing, about 0.5 mm. bore) is mounted in front of the supporting board and

carries a longitudinally split meter stick on its sides. On this is mounted a slide rule index *b*, the cross hairs of which may be adjusted on the mercury meniscus, thus making accurate reading easy and avoiding parallax. A stopcock 'c' on the top of the tube provides for filling the burette. The stopcock is filled with the titrating solution, the mercury run up to the tube junction, the stopcock opened and the mercury dropped, the liquid following down the tube. Gravity fills the delivery side of the burette tube. 'd' is a device for interchanging capillary tips, either on account of breakage or in order to deliver the titrating solution at varying angles to suit the type of titrating vessel and other arrangements of the apparatus. It consists of two brass collars with an open-sided coupling between, which draws the collars together. These collars are fastened to the tubes with de Khotinsky cement. The ends of the glass capillary tubing which join inside the coupling are ground square and a little rubber cement applied to the ground surfaces makes a satisfactory gasket."

The control of the burette is very efficient and the setting of the mercury meniscus may be made as close as can be read. It is pointed out that the chief disadvantage is the fact that some types of solutions oxidise the mercury and deposit a residue in the tube. For this reason Kirk also describes another burette in which a small air space exists beneath the mercury thread and the fluid.

In the calibration of the above burette the variation in the bore of the tube is determined by measuring the length of a short mercury thread over successive intervals of the tube. The average volume delivered per scale division is measured by weighing a delivery of water in a small, tightly stoppered vessel. To the average volume of water delivered per scale division, the corrections found for bore variation by the mercury thread are applied.

*Titration table.* To avoid or diminish breakages a titration table is used by Kirk, (Fig. 13) made of wood painted white and having the dimensions  $2\frac{1}{2}$  by  $4\frac{1}{2}$  inches. This is raised and lowered by the mechanism shown in diagram, 'a' being the coarse and 'b' the fine adjustment. With this mechanism

the table is raised to approach the fine tips of the stirrer and burette, and the movement may be made as delicate as possible.

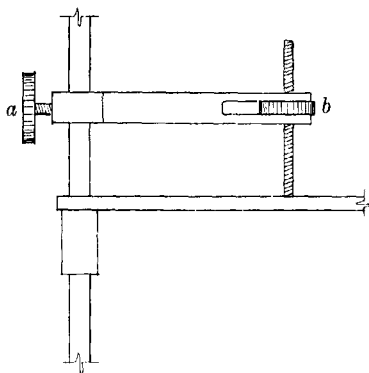


FIG. 13. Arrangement for support of titrating table with coarse and fine adjustment as described by Kirk (11).

**Technique of Linderstrøm-Lang and Holter (25, 26).** The method adopted by Linderstrøm-Lang and Holter for ammonia absorptions and titrations (26), and appearing shortly after the description of the standard micro-diffusion apparatus or Unit (1) indicates the lines on which the principle may be applied in a different way to drop scale determinations. The method adopted by them for the absorption is of an ingenious kind but not ideal, since they required 5 hours for the full absorption even at  $40^{\circ}$ , arising from the stratum depth below the diffusing surface. Their micro-diffusion arrangement is of the following kind. At the bottom of a small tube (25/3.8 mm., 6 mm. outer diameter) lined with paraffin is placed a drop of fluid for analysis (about 14 c.mm.). Into the tube, held horizontally, is then introduced approximately 7 c.mm. of 2N NaOH about 5 mm. above the bottom of tube, and 35 c.mm. of water about 5 mm. from the top. On slanting the tube the drops are run together, the water drop preventing the escape of ammonia, until 7 c.mm. of hydrochloric acid is introduced towards the upper part of tube. The tube is then ready for the ammonia diffusion and equipped with a cap as in

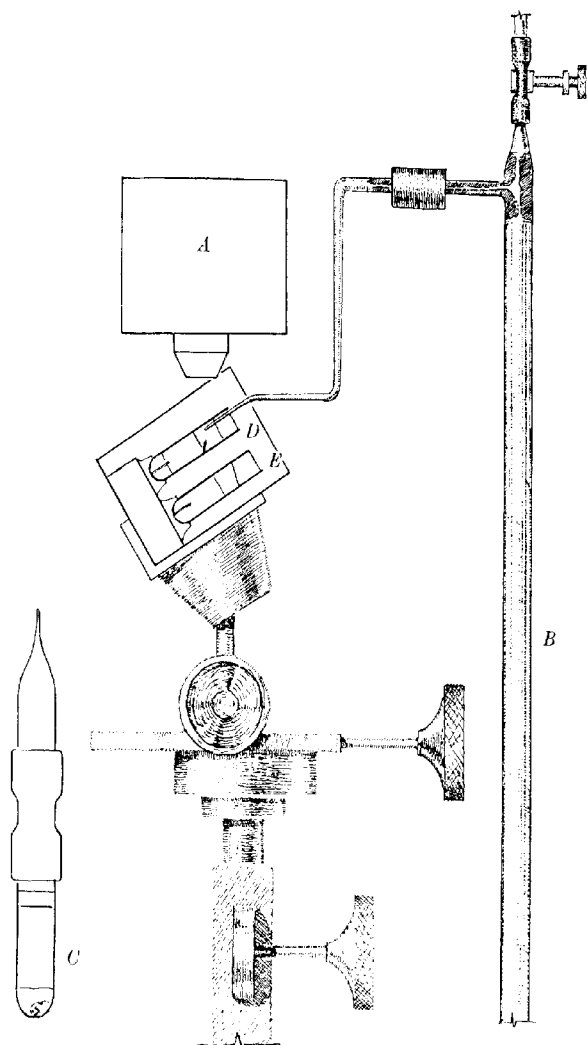


FIG. 14. The completed absorption tube (C) and titration arrangement from Linderstrom-Lang and Holter (*Z. physiol. Chem.*, **220**, 5). The magnet *A* is used to move the stirrer in the drop of acid in the upper portion of tube *D*. *Vide text*.

Fig. 14, *C*, being introduced into its holder in a thermostat at 40° C. After about five hours the absorption is complete and the acid is titrated with *N*/10 NaOH or *N*/100 borate from a burette on the Rehberg principle (with Widmark and Ørskov's modification, 27), to a *pH* of 5.9, the end-point being judged by the presence of a companion tube as in Fig. 14 (*E*), with acid containing the indicator, buffered with phosphate at 5.9 and introduced in a similar way.

In the above procedure it is necessary that both the alkalinised fluid and the titrating fluid be stirred, and an interesting procedure has been used for the purpose. The stirrers consist of small capillary tubes (1.1–1.5 mm. long) filled with Ferrum Redactum and fused to form small pear-shaped bodies. One of these is introduced (after testing) into the fluid and moved by a magnet (*A*, Fig. 14) externally placed, the magnet current being broken periodically by a metronome arrangement.

The stirrers are tested after making by boiling in dilute nitric acid, and subsequently testing this with potassium thiocyanate.

In the above procedure it will be seen that the fluid depth at the bottom of the tube is altogether about 56 c.mm., giving with an internal diameter of less than 3.8 mm. (some of the 3.8 mm. diameter being taken up by the paraffin lining) a depth of about 5 mm. From the principles of Chapter IV this should give from the equation

$$t = 1230a^2$$

$$= 307 \text{ minutes or } 5 \text{ hours}$$

—which corresponds to the time allotted by Linderstrøm-Lang and Holter. With respect to the exclusion of atmospheric carbon dioxide and the use of bromocresol purple, this is unnecessary for the kind of titrations used. As shown in Fig. 9, the glass error on titrating with methyl red with full exposure to carbon dioxide begins to be affected by the variable chemical error only in the region of *N*/1000. The constant error of the titration to a *pH* of 5.5 need be of no consequence in itself, the ammonia absorbed being determined by a subtraction of two titrations to the same end-point. What signifies is the variable error in judging the end-point colour, which

is reflected in variations of titrating alkali related to the total buffering of hydrogen ion, of indicator and atmospheric carbon dioxide at the end-point. This is fully discussed in Part III.

*Pipettes used.* The kind of pipette used by Linderström-Lang and Holter (25) for introducing small drops into the tubes already described, is shown in Fig. 15. The pipette itself (*A*) is

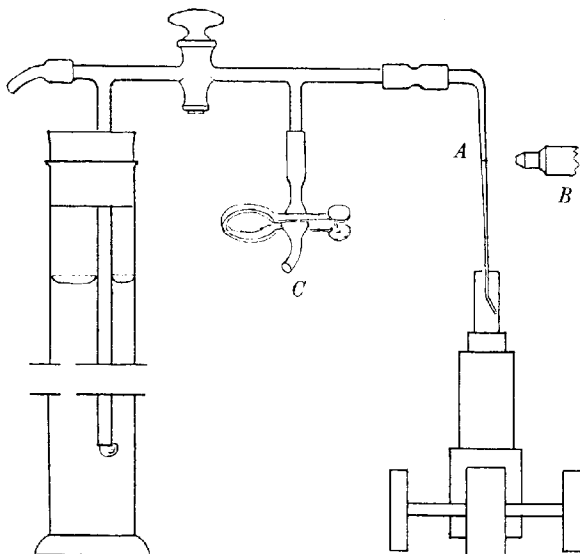


FIG. 15. Arrangement for fine pipetting (order of 10 c.mm.) by Linderström-Lang and Holter (from *Zeitschr. für physiologische Chemie*, 201, 1931).

a capillary tube drawn out to a fine tip with a slight bend at the end, which serves the purpose of delivery against a wall surface without fear of contact by the body of the pipette. The pipette, the end of which dips into the fluid for analysis is filled somewhat above the mark by sucking through tube, the clip being then replaced. The meniscus falls slowly and is observed by the reading microscope at side (*B*); when the meniscus has exactly reached the mark the support of supply tube is suddenly lowered by twisting on its screw and the fluid is then held in the pipette by capillary forces. The contents of the pipette are



ejected by the arrangement shown in Fig. 15, the tap being opened and a pressure of about 20 cm. water allowed to expel the drop, within about 5 seconds. Such pipettes may be standardised on a micro-balance, but the wall fluid should be allowed for, and this may be done by the formula in Chapter V.

Alternatively they could be standardised by a titration procedure, in which a delivery of  $N$  HCl is titrated with  $N/100$  or  $N/200$  alkali, from a micro-burette of an ordinary type.

*The burette.* The burette used is of the Rehberg type with the modification of Widmark and Ørskov (27), in which the volume of fluid expelled is measured by the amount of turn of the screw, which is graduated for this purpose.

## CHAPTER VIII

### COLORIMETRY IN THE MICRO-DIFFUSION METHODS

THE colorimetric methods (for minute halogen concentrations) described in Part II may be suitably modified for any kind of accurate micro-colorimetric technique in which volumes of the order of 1 ml. are dealt with. It has been found most suitable, however, to use the spectrophotometric procedure. This has been used also by Borsook (28) in the determination of minute ammonia and total nitrogen, using an absorbing Unit similar to that described in Chapter II. The colorimetric methods described in Part II were originally worked out with the Leitz triple stage dipping colorimeter with spectrum filters and the grey solution of Thiel (29), but may be carried out with advantage using the newer Leifo instrument of Leitz, which dispenses with the grey solution, or by means of the Pulfrich photometer. Here it is proposed to indicate chiefly for the students' use only the general principles of such colorimetry or spectrophotometry.

**Principles of colorimetry, including spectrophotometry.** In all the simpler colorimetric methods of analysis the concentration of a coloured substance in solution is determined by comparing the depth with that of a known concentration of the substance which gives to the eye the same colour intensity. The instrument or colorimeter in which this comparison is made is now most generally constructed on the Dubosc principle, illustrated in Fig. 16. In this two cylindrical vessels contain the solutions for comparison and have the under surface of plane glass. Through variation in the depths of the cylindrical glass plungers  $P$  and  $P_1$ —the positions of which are indicated by a scale at the back—the light reflected from a mirror is made to pass through different strata of the coloured fluids. Passing up through the plungers, this light enters the prisms  $p$  and  $p_1$ , thence through a lens system into the eye of

the observer, who sees the light from each coloured stratum as a corresponding half of a circular field of vision. On varying

the position of the plungers a series of matches can be made in which both halves of the field of vision appear of equal intensity.

In any such match we may suppose that the light on each side has passed through the same number of molecules of the coloured substance. Then if the layer of the unknown concentration is 10 mm. and that of the known 20 mm., it may be assumed that the concentration in the solution for analysis is twice that of the standard solution, or generalising,

$$\frac{c}{c_1} = \frac{d_1}{d} \quad \text{or} \quad c = \frac{c_1 d_1}{d},$$

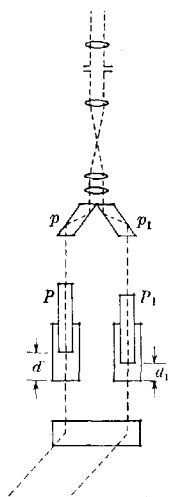
in which  $c$  is the concentration of the observed solution and  $c_1$  that of the standard,  $d_1$  and  $d$  being the corresponding stratum depths giving equal light intensities.

FIG. 16. Construction principle of the Duboscq colorimeter. (From Pincussen's *Mikromethodik*, Thieme, Leipzig.)

Actually in the above equation we make four different assumptions, at least three of which need not necessarily be true. It is supposed :

(1) That the absorption of light per unit distance through a stratum of uniform concentration and temperature is proportional to the light intensity at any level of the stratum. The truth of such an assumption is expressed as Lambert's law. If it were incorrect the ratio of the strata giving equal light intensity would not be as 2 : 1, with a concentration ratio of 1 : 2.

(2) It is assumed that the absorption of light through any stratum is proportional to the concentration of the absorbing or coloured substance. The truth of this assumption is expressed as Beer's law, or both (1) and (2) may be grouped together as the Lambert-Beer law.



It not unfrequently happens that Beer's law does not hold strictly, and occasionally the deviation therefrom is gross.

(3) It is supposed that the coloured substance is the only substance dissolved in exactly similar solvent in both solutions, or that any extra material does not affect the optical properties of the coloured substance or solvent. This is seldom strictly fulfilled, but often sufficiently for the most accurate analysis. Gross errors can arise by assuming the similarity of solvents when in fact suspended material or a second coloured substance may be present in the solution investigated.

(4) It is supposed also that the solvent is not itself coloured and that under the colorimetric conditions it does not absorb any appreciable amount of light.

Difficulties arising from points (3) and (4) are generally removable by the procedure of compensation. How this is applied may be seen from Fig. 17, showing the principle as used in the Leitz colorimeters.

$A$  contains a solution of the substance in water of known concentration and  $A_1$  contains water. The plungers in  $A$  and  $A_1$  move together as also those in  $B$  and  $B_1$ . When a match is made it is obvious that a compensation is

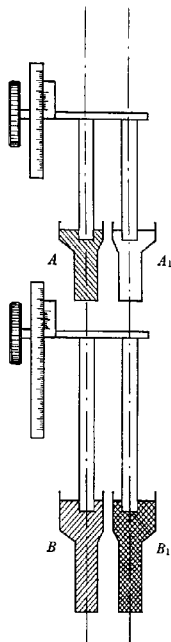


FIG. 17. The figure shows the manner of compensation for coloured solvent as used in the Leitz instruments. If a coloured substance is dissolved in varying concentration in a coloured solvent of constant or known colour value, then the above arrangement in which  $B$  is the coloured solvent,  $B_1$  the same containing the coloured substance,  $A$  a standard solution of the substance and  $A_1$  distilled water, the effect of the solvent is eliminated. This follows from the fact that in any colour balance the same depth of coloured solvent is looked through on each side.

likewise effected, the light passing through the same amount of water and of solvent on both sides.

*Deviations from the Lambert-Beer law can only be avoided in ordinary colorimetry by having the concentration of the standard solution close to that of the observed solution, and in general it is found inadvisable to have it exceeding the range of 0.3 to 3 times.*

Such difficulties or restrictions are removed in the practice of spectrophotometry.

**Monochromatic light and spectral filters.** Monochromatic light may be used in ordinary colorimetric work with advantage. Such light could be introduced at the light source, but it is simpler and equally effective to remove all but the given wave band by a spectral filter inserted, as in Fig. 18, in the path of the mixed rays after absorption and before entering the eye. The spectral filter is a small circular disc of coloured glass with uniform thickness. A number of such (about 10-12) covering the visible spectrum are mounted on a disc, which can be moved alternately into position. These filters allow light to pass of maximum intensity at some particular wave-length and with diminishing intensity on each side, so that at a range of the order of 40  $m\mu$  the intensity is about one-tenth that at the centre.

In order to secure a readily measurable absorption of light, a filter is chosen which has a colour towards the opposite side of the spectrum to that of the coloured solution, or more specifically one which corresponds to the region of maximum absorption.

Even with ordinary colorimetry the use of monochromatic light secures the following advantages. It simplifies the visual judgment, since on moving the plunger up and down the colour observed is darker or brighter, the judgment being concerned only with luminosity, though of a particular colour. With ordinary lighting as the stratum depth increases the total luminosity decreases, but the colour observed appears to grow in intensity. It also allows a certain range of colours to judge from, and we can change a comparison of yellow lighting to one in the blue or green region of the spectrum. Furthermore, it will occasionally permit the elimination of an interfering

coloured substance by the use of a wave band in which this shows no absorption.

Beer's law will be also more strictly fulfilled using monochromatic light.

**Points in the use of the colorimeter.** Before use the cups should be well cleaned, particularly the underneath surfaces, and the plungers, etc. freed from dust. In the more modern instruments the prisms are inclosed and protected, as also the front of the colorimeter when not in use or when a comparison is being made. The requisite cups are then filled with the observed solution and the standard, the arrangement in Fig. 17 being adopted when the compensating device is used. All cups not containing standard, observed solution or solvent, should be removed if this can be done similarly on each side or else contain distilled water in which the plungers are immersed. The plungers in the observed solution and standard are now turned down until they touch the glass. If the scale readings are not exactly zero they should be adjusted thereto. With the plungers at zero the eyepiece is focused so that the dividing line is made as sharp as possible and the lighting arranged until equal luminosity appears in both halves of the field.

The adjustment is made with the front of the instrument protected with its shutter or alternatively in an evenly illuminated part of the room. The lighting is very easy to regulate in the newer instruments with the lamp incased, fixed to the instrument and provided with centring or other adjusting devices.

In making the comparison, depths below 5.0 mm. should be avoided if possible. There appears to be a difference of opinion as to the most suitable number of readings to take with the one solution, and the author prefers to take at least five. When the reading of one solution is finished it is essential that the lighting be again adjusted with the plungers at the zero position.

In using micro-cups (1 ml.) a small air-bubble is occasionally trapped underneath the plungers and can give rise to very erroneous readings. This should be borne in mind when lowering the plungers into the solutions.

**Spectrophotometry.** This is the measurement of the rela-

tive diminution in intensity of monochromatic light on passing through a coloured or absorbing stratum.

We may consider in the colorimetry previously described that a spectral filter is in use, then if instead of the standard solution and plunger on one side we had some means of progressively and measurably diminishing the light until a match is obtained with a given stratum depth on the other side, we could obtain a measure of the ratio in which the light intensity for a given wave band has been diminished by the coloured solution. This would exemplify the principle of spectrophotometry, and different methods have been used for producing and measuring the light diminution. In the simpler types of spectrophotometers in use to-day for analytical work the light intensity is diminished by a progressive constriction of the light orifice, though this in turn allows a variety of applications. The use of polarised light and the rotation of a Nicol prism has also been used in physical investigations. Instead of such devices for diminishing monochromatic light in a measurable way over the visible spectrum, the same effect could be produced by retaining the Duboscq principle but using instead of standard solutions of the substances investigated, one which has a constant absorbing power over the whole spectral range. Such a universal standard is approximated to by the *grey solution* of Thiel.

Using these spectrophotometric methods deviations in the application of the Lambert-Beer law are of no consequence, when after an initial observation of the light diminution in unit stratum the concentration is read off from a curve originally constructed for solutions of known concentrations. We not only secure this advantage, but after such a curve is drawn, any further labour in the making of standards is removed.

In the actual plotting of such curves the relative diminution of light intensity is not used directly, but rather a function of this termed the extinction coefficient, for it is the extinction coefficient and not the light intensity that goes in linear proportion to the concentration when the Lambert-Beer law applies. How these quantities are related is considered in the following account.

**Relations of light intensity, extinction coefficient and stratum depth.** If we suppose radiant energy such as monochromatic light passing through a coloured fluid, as in a colorimeter cup, with the incident rays perpendicular to the fluid surface, then we may write

$$-\frac{di}{dl} = ik, \quad (16)$$

where ' $i$ ' is the intensity of the incident light, ' $l$ ' the length of the absorbing layer and ' $k$ ' a constant expressing the absorptive property of the substance in solution.

Lambert's law is expressed by this equation. That the absorption per unit length (measured over a very small or infinitesimal layer) should be proportional to the amount of radiation entering the layer may be explained if we suppose the radiation as made up of units which are absorbed when they impinge directly on molecules, total absorption being therefore proportional at any time to the 'concentration' of the radiation.

The relation may be further expanded to

$$-\frac{di}{dl} = ick', \quad (17)$$

in which form it may be termed the Lambert-Beer law. Here the absorption per unit length is not only proportional to the radiant energy entering, but also to the concentration of the absorbing molecules, which should apparently follow from our first supposition of moving molecules being struck by the radiation units, since doubling the molecular concentration doubles the number of molecules struck. This at least forms an easy if figurative interpretation, though as noted, the Lambert-Beer law is not always strictly obeyed (for example, it is not valid in the presence of proteins, as in blood serum).

From the first equation we obtain on integrating

$$-\log \frac{i}{i_0} = lk \times 0.434, \quad (18)$$



and if the intensity of the incident light is regarded as unity, then

$$\begin{aligned} -\log i &= lk \times 0.434 \\ \text{or} \quad i &= 10^{-lE}, \end{aligned} \quad (19)$$

(where  $E = k \times 0.434$ ).

For different concentrations of the same substance and with incident light of unit intensity, then when the depths are adjusted to produce the same intensities, from equation 19

$$\frac{E_1}{E_2} = \frac{l_2}{l_1}, \quad (20)$$

From equations 16 and 17 and writing  $E$  again for  $k \times 0.434$ , as in equation 19, we have  $0.434ck' = 0.434k = E$ , from which and equation 20

$$\frac{E_1}{E_2} = \frac{c_1}{c_2} = \frac{l_2}{l_1}. \quad (21)$$

which is the fundamental relation of colorimetry and spectrophotometry.  $E$  is termed the *extinction coefficient* and  $\frac{E}{c}$  (or  $E_0$ ) the *specific extinction coefficient*. The extinction coefficient was originally defined by Bunsen as the reciprocal of the depth which absorbed 90% of the incident radiation (with 90% absorption we may write

$$\begin{aligned} i &= 10^{-1} = 10^{-lE} \\ \text{or} \quad E &= 1/l, \end{aligned}$$

which accords therefore with the original definition).

It is convenient to define  $E \times l$ , the extinction coefficient multiplied by the stratum layer, as  $E'$  or the 'extinction';  $E$  is then given by  $\frac{E'}{l}$ . It is the  $E'$  values or 'extinctions' that are marked on the drum of the Pulfrich photometer.

It will be seen that *we can associate such extinctions or extinction coefficients directly with the concentrations to which they are simply proportional, whereas this is not so with light intensities.*

**Determination of concentrations by measurement of extinction coefficients.** The relation of the extinction (as defined

above), the extinction coefficient and the specific extinction coefficient may be written

$$\frac{E'}{l} = E = E_0 c. \quad (22)$$

Since the specific coefficient is a constant for a particular substance and given wave-length and may be determined once and for all, it follows that any method or instrument whereby we can determine  $E'$ , or  $E$  for light of fixed wave-length, gives us a means of determining concentrations without standards.

(a) *The 'grey solution' of Thiel and spectral filters (Leitz).* The 'grey solution' of Thiel (now made up from the solid substance) has an extinction coefficient of 0.50 over the visible spectrum. It may be used with any simple colorimeter which can be provided with filters, if these are not already fitted. Fig. 18 illustrates its use. (Fig. 18a shows the two-stage colorimeter with filter equipment.)

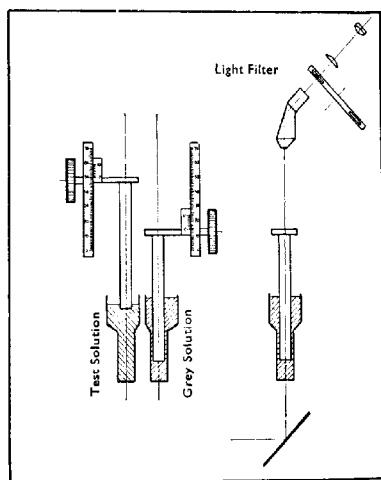


FIG. 18. Diagram illustrating use of the 'grey solution' for determining extinction coefficients (from *Leitz Bulletin*, No. 4/micro).

With the grey solution in one cup and the solution for analysis in the other, then if we use a filter with wave-band

around  $464\text{ m}\mu$ , we shall see both fields blue in colour, and may adjust readily to an equal depth of blue. The extinction coefficient is then given by

$$\frac{E_s}{E_g} = \frac{l_g}{l_s}$$

or  $E_s = 0.5l_g/l_s$ , (23)

where  $l_g$  is the depth of grey solution and  $l_s$  that of the given solution. If the depth of the latter is made 0.5 cm. the reading

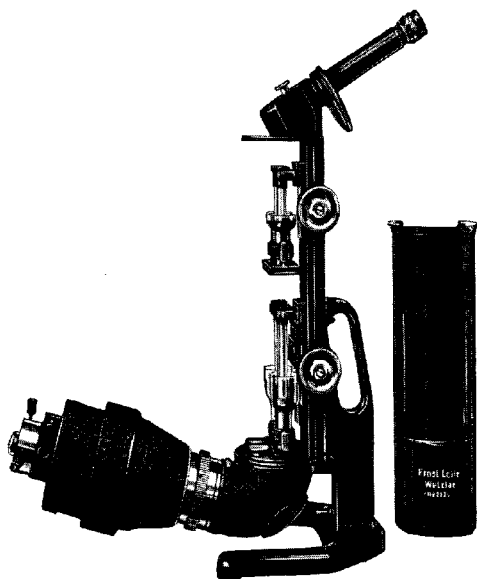


FIG. 18a. Two-stage universal colorimeter (Leitz) with lamp attached and revolving disc carrying 11 spectral filters.

of the grey solution gives at once the extinction coefficient. The simplest manner of using the grey solution for determining concentrations is as follows. In this some comparisons of the corresponding depths of the grey solution with that of a standard solution are made with a given filter, the depth of the grey solution being placed at some figure which has a simple

numerical relation with the concentration of the standard, the corresponding depth of standard solution being noted. Subsequently the 'unknown' solution is placed at once at this depth and the reading of the corresponding grey solution depth gives the concentration in a simple relationship. For example, if 1 ml. of  $N/1000$  HCl is oxidised in the outer chamber of the standard Unit and after absorption in the 20% iodide this solution were placed in one cup and the grey solution in the other at a depth of  $\frac{35.5}{2}$  or  $\frac{35.5}{3}$  (using filter No. 3, Leitz), then the corresponding reading of the iodide solution gives the standard figure necessary. On placing the depth of subsequent unknown solutions at this value the corresponding grey solution depth multiplied by 2 or 3 gives the chlorine analysed as  $\gamma$  chlorine.

#### Determination of extinctions by the Pulfrich Photometer.

A special principle of the Pulfrich photometer may be seen from Fig. 19, in which by means of two opposed screws con-

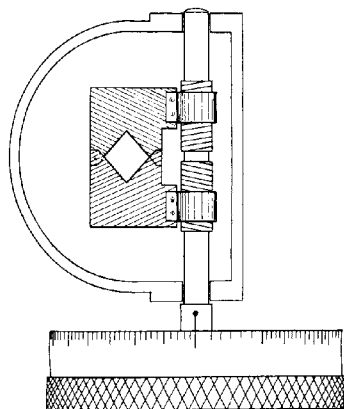


FIG. 19. Diagram illustrating an essential principle of the Pulfrich photometer.

trolled on the one axis by the movement of a graduated drum, the diagonal of a square opening may be progressively dimin-

ished and in direct relation to the angular rotation of the drum.

If similar monochromatic light passes through water and a coloured fluid contained in opposite cells, then the emergent light will appear of the same colour, but of different intensities. If this emergent light passes through two such similar square apertures, it can be reduced on either side to form equal intensities. On the side containing the coloured fluid the aperture may be fully open or at  $360^\circ$  angle on drum, the required rotation of the other drum giving a measure of the light absorption and hence the extinction of the coloured fluid.

Fig. 20 shows how this principle is rendered optically effective in the Pulfrich photometer, the optical components of which

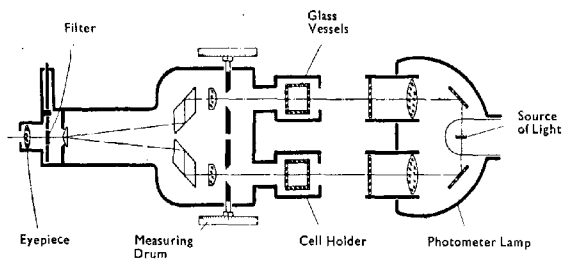


FIG. 20. Diagram of optical arrangement in the Pulfrich photometer.

comprise a monocular double telescope set at infinity with an axial separation of 70 millimetres. The emergent light from the two cells and the drum-controlled aperture passes through two object lenses, the two beams being afterwards combined into one field of view in the eyepiece by the two separate prisms and the single biprism. The setting is arranged so that the optical axes of both telescopes and hence the aerial images of the square apertures of entry coincide. The images of both the apertures of entry are seen, on looking into the inspection eyepiece placed in front of the ordinary eyepiece, as bright square apertures. Looking through the ordinary eyepiece the two images are received by the pupils of the eye in such a way that two semicircles appear representing each half of the field of vision with a sharp line of demarcation on focusing.

The light in each half-field represents the emergent light from the corresponding absorption cell and the spectrum filter. Fig. 20a shows the optical bench assembly of the Pulfrich photometer.

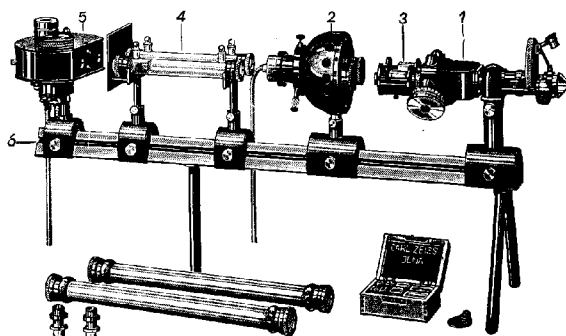


FIG. 20a. Pulfrich photometer (1); with Photometer lamp (2); cells in cell holders (3); absorption tubes (4); turbidimeter (5); and optical bench (6).

**Relation of drum rotation to light intensity and extinction coefficient.** The intensity of the emerging light from the square aperture is proportional to the surface, hence to the square of the diagonal and consequently to the square of the angular rotation, since each angle of this corresponds to an equal increment of aperture diagonal. We have, therefore,

$$i = \left( \frac{a}{360} \right)^2 \times \text{a constant.} \quad (24)$$

We can regard the intensity as passing from 100% to zero as the aperture is fully opened and closed, so that when  $a$  is  $360^\circ$ ,  $i$  is 100, and therefore

$$i = \left( \frac{a}{360} \right)^2 \times 100. \quad (25)$$

This relation of intensity to  $a^2$  rather than  $a$  ensures much greater drum movements for equal concentration increments at the lower levels. The drum is graduated in percentage

intensities, and in the newer models in extinctions also. The extinction value is given by

$$\begin{aligned}\log \frac{100}{i} &= E' \\ &= El \\ &= E_0 cl,\end{aligned}\tag{26}$$

where  $E$  is the extinction coefficient,  $l$  the stratum layer,  $E_0$  the specific extinction coefficient and  $c$  the concentration.

Reading  $E'$  directly on the drum we obtain  $E_0 cl$

$$\text{or } \frac{E'}{E_0 l} = c \text{ (as g./litre).}\tag{27}$$

When Beer's law is not obeyed the concentration must be determined from a curve of extinctions against concentrations. Such curves may be used generally and are particularly useful for large serial determinations.

**The range of error in the determination of concentration.** For such absolute colorimetry or photometry, using the dipping colorimeter with grey solution, or the Pulfrich photometer, a difference of intensity in the two halves of the fluid of vision can be detected, with some experience, when one field is about 1 to 2% lighter than the other.

From this we may calculate the following table :

TABLE IV

Intensity of transmitted light. $i$ (— % of $i_0$ ).	Extinction $E'$ ( $= \log \frac{100}{i}$ ) and proportional to the concentration).	Percentage error in concentration with an error in light discrimination of	
		1%	2%
5	1.301	0.3	0.7
10	1.000	0.4	0.9
20	0.699	0.6	1.2
50	0.301	1.5	2.9
80	0.097	4.4	8.9

(A somewhat similar table has been compiled by Weigert (30), as quoted by Heilmeyer (31)).

The most efficient range lies between 10% intensity (90% absorption) and 50%. Below 10% the field is becoming too dark, and error from this source increases more rapidly than that of the lessening of the relative percentage error. Higher than 50% the percentage error is too high for accurate working.

These intensities correspond to extinctions of 1.0 and 0.3 respectively and to 20 mm. and 6 mm. depth of grey solution, with the Leitz dipping colorimeter, and to  $114^\circ$  and  $255^\circ$  with the Pulfrich photometer, which is roughly one-third to two-thirds the whole drum rotation.





## PART II

### DESCRIPTION OF METHODS WITH THE STANDARD UNIT

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#### CHAPTER IX

##### AMMONIA. GENERAL METHOD

THE ammonia group of determinations includes not only ammonia itself, but substances which yield ammonia after the action of ferments easily procurable and relatively stable. Such are urea, adenylypyrophosphoric acid, adenosine and adenylic acid. The method is also suitable for micro-Kjeldahl analyses and for macro-Kjeldahl determinations of total nitrogen, and can give an accuracy for the ammonia determination not less than that with the usual 25 ml. technique. There should be no difficulty experienced in attaining accuracies of the order of 0.5% as a coefficient of variation.

**General method for ammonia determination (acidimetric procedure).** The method which the author has investigated most extensively and found very satisfactory consists in the absorption of the ammonia in hydrochloric acid and subsequent titration with barium hydroxide, which latter is always standardised against the acid by preliminary titration. The same description applies with minor differences to all ranges of ammonia determination down to 0.1 $\gamma$  (0.0001 mg.) ammonia nitrogen with the standard Unit. The range is suitably covered by three standard acid and alkali solutions whereby 90 to 0.1 $\gamma$ N may be determined with the maximum available accuracy for end-point volumes of the order of 1 ml.

Where the amount exceeds 90 $\gamma$  per ml. the fluid may be diluted or a smaller volume taken, since there is practically no advantage from the point of accuracy in the determination of larger quantities.

*Delivery of the acid.* Into the <sup>inner</sup>outer chamber of a Unit cleaned according to the procedure described on page 10, is run 1 ml. of the standard acid containing indicator (Solution I). The acid is delivered from an Ostwald, simple pyrex tube pipette, graduated 1 ml. pipette, or the 2 ml. standard burette, as described in chapter V. For the finest work the simple tube pipette with slow delivery and comparatively fine point is recommended. Where less than  $N/500$  acid is required for the central chamber, the simple pyrex pipette should be used, as previously mentioned. Help in the choice of suitable acid and alkali strengths will be obtained from Table V.

TABLE V. *Suitable solution strengths in determining ammonia with the hydrochloric-barium-hydroxide procedure*

Range.	Acid in central ch.	Solution in burette.	Each large div. on burette (0.01 ml.) as $\gamma N$ .	Max. ammonia $\gamma N$ .	Applications.
I	1 ml. $N/150$ hydrochlor. ac. ( <i>Vide p. 79.</i> )	35.7 ml. $N/10$ bar. hyd. diluted to 250 ml.	2.0	93.4	All accurate ammonia determs. beyond about $14\gamma N$ . Useful for urine ammonia.
II	1 ml. $N/1000$ hydrochlor. ac. ( <i>Vide p. 79.</i> )	21.4 ml. of $N/10$ bar. hyd. diluted to 1000 ml.	0.30	14.0	Various tissue ammonia determs. Micro-Kjeldahls, etc.
III	0.7 ml. of $N/5000$ hydrochlor. ac. ( <i>Vide p. 79.</i> )	Sols. of Range II diluted five times	0.05	1.96	Blood and tissue ammonia.

The above solution strengths are given as general guides, though many others will no doubt be found useful for the particular purposes in view.

The acid introduced into the central chamber is very conveniently made up for use as described in the text.

When the ammonia to be determined lies beyond 0.070 mg.  $N$ , the solution may be diluted to fall within range I. No appreciable increase in accuracy will be obtained by using stronger solutions.

It is essential that the acid should cover the full surface of the central chamber, but no difficulty will be found here if the Units have been properly cleaned.

*Delivery into the outer chamber.* For delivery into the outer chamber, volumes may range from 0.1 to 2.0 ml. (or nearly 3.0) with the standard Unit. These may be introduced as described in the previous chapter, i.e. either by the Ostwald or the graduated pipette or by a simple tube pipette, and here again the simple tube pipette is to be preferred for the finest work. To prevent spattering vigorous blowing should be avoided. Washing out of pipette is to be avoided except for the smallest deliveries (0.1 to 0.2), and then only once or twice, owing to the effect of volume increase on absorption times.

*Covering with lid.* The lid is smeared with the fixative (described below) and placed on the Unit. It is just as convenient to smear the whole lid surface as part thereof.

*Liberation of the ammonia with alkali.* The Unit is tilted by resting on a spare lid and the lid displaced just sufficiently to allow the introduction of the tip of a pipette. 1 ml. of saturated potassium carbonate is then usually introduced by a simple tube pipette with rapid delivery. The delivery of the saturated carbonate need be only very approximate, since if less than 1 ml. is delivered there is less volume in the outer chamber, and if greater the mixture is more concentrated in the carbonate, the effects tending to balance out. At the same time it is essential that the carbonate solution be saturated or very close thereto and it is always advisable to have some solid carbonate in evidence in the stock solution. *Sodium carbonate cannot be substituted for the potassium salt unless much longer times are allowed for the absorption.* Alkaline fluids other than potassium carbonate may be found more suitable for special determinations—particularly where the ammonia-containing fluid is already strongly acidic, in which case saturated potassium metaborate or 50% sodium hydroxide may be used.

*The absorption period.* This varies with the fluid volume that is present before introducing the 1 ml. saturated carbonate

as shown in Fig. 21. The figure gives the full absorption periods. (In the original account minimal absorption times were given from equation 3, ref. 1.)

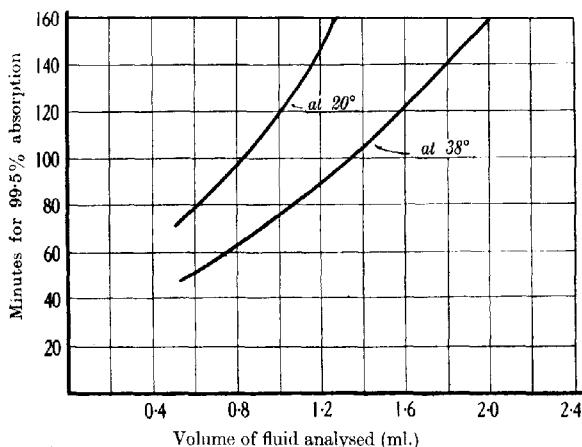


FIG. 21. Times for full (99.5%) absorption in unit, when to varying amounts of fluid in the outer chamber 1 ml. of sat. potassium carb. is always added.

The times have been deliberately increased beyond the minimal times of the original account to ensure full absorption.

*Titration.* The acid in the central chamber of the Unit is now titrated with one of the three barium hydroxide solutions of Table V, according to the range. The end-point may be taken as the complete disappearance of the reddish colour, or the first appearance of a faintly perceptible green ( $pH=5.5$ ). A thin pyrex glass rod should be used for stirring. It is essential that a blank determination be done for the smallest ranges since minute quantities of ammonia may be present in the fixative, etc.

*Calculation of results.* If acid and alkali strengths are those of Table V the calculation of the results becomes very simple, the number of large divisions on the burette being multiplied or divided by simple numerals.

An example may be useful to illustrate the calculation. In this example—as throughout for similar calculations—the Unit

containing the fluid and all the required reagents is termed the 'analytical Unit,' in contrast to the 'blank Unit' set up as described in the special determinations.

EXAMPLE. Solutions of Range II used (Table V).

1 ml. of  $N/1000$  acid in central chamber.

0.5 ml. of fluid for analysis in outer chamber.

*Titration.*

Analytical Unit - - - 36.75 large divs.

Blank Unit - - - 55.30 " "

*Calculation.*

$$(55.30 - 36.75) \times 0.30 = 18.55 \times 0.30 = 5.56\gamma N,$$

$$\gamma \text{ ammonia } N/100 \text{ ml.} = \frac{5.56}{0.5} \times 100 = 1112$$

or mg. ammonia  $N/100$  ml. = 1.112.

The result must be corrected for pipette and alkali factors when these differ from the nominal volume or strength.

#### Solutions required.

(1) *Standard hydrochloric acid with indicator.* To cover all ranges three strengths of standard acid are made up (see Table V). One of these is used depending on the range expected. The three acids are  $N/150$ ,  $N/1000$  and  $N/5000$  hydrochloric acid. (Sulphuric acid could also be used with no appreciable difference at these dilutions.) In preparing these stock acids  $N/10$  and  $N/100$  should be firstly made up from standard normal acid. In preparing the standard acids with indicator, we will suppose that 500 ml. is the quantity made up in each case.

*$N/150$  HCl with indicator.* 5 ml. of Tashiro's reagent are run into a 500 ml. flask and 100 ml. of absolute alcohol are added. Distilled water is then run into about three-quarters the volume of the flask and the indicator brought to the neutral point, which usually means dropping in a little dilute alkali until the red colour is discharged. 33.3 ml. of the stock  $N/10$  are now run in from a burette and the mixture brought to the mark, etc. The mixture is stored in a pyrex bottle or an ordinary bottle lined with solid paraffin. It will keep practically indefinitely. The distilled water used should be tested for its reaction by some of the Tashiro indicator and—at least for the two weaker acids—should be neutralised to the end-point if

necessary. Tashiro's reagent is made for the above purpose by adding to 200 ml. of 0.1% alcoholic solution of methyl red, 50 ml. of a similar solution of methylene blue (0.1%). Stored in a brown bottle it keeps indefinitely.

*N/1000 HCl with indicator.* The same procedure is followed exactly except that 5 ml. of the stock *N/10* acid from a pipette is used instead of 33.3 ml.

*N/5000 HCl with indicator.* Here again the same procedure is adopted except that 10 ml. of the stock *N/100* acid is used.

(2) *Standard barium hydroxide solutions.* These are prepared from the stock *N/10* barium hydroxide as supplied by the British Drug Houses, and in accordance with the dilutions in Table V. The barium hydroxide stock solution may be run in from a standard 50 ml. burette. All undue exposure to the atmosphere should be avoided, and after making to the mark and mixing, the solution should be transferred to the reservoir bottle of the burette. Before a titration series the alkali is always standardised against the acid.

For the weaker solutions it is advisable to paint the cork of the reservoir bottle with melted paraffin wax, including the connections with the soda-lime tube, to prevent slow diffusion of atmospheric carbon dioxide into the solution.

(3) *Saturated potassium carbonate.* A very large amount of potassium carbonate is required to saturate water (53.2 g. of  $K_2CO_3 \cdot 2H_2O$  being contained in 100 g. of the solution at 25° and 60.9 g. at 100°—ref. 33). Excess should be added, and it is convenient to weigh out 110 g. per 100 ml. water. After dissolving in a large beaker and cooling (it heats on mixing with the water) the saturated solution is decanted into a pyrex conical flask with pyrex stopper, some glass beads are added and the solution boiled for about ten minutes at first vigorously and then gently. The stopper is then placed in position with a small fold of filter paper inserted between it and the flask to allow the air to enter in cooling and prevent the stopper from sticking. After cooling, a little solid carbonate should be in evidence. This boiling of the carbonate solution is necessary to free it completely of ammonia, for potassium

carbonate generally contains some small quantity of free ammonia.

*The fixative.* The fixative for smearing on the lid when using the Unit with the  $N/150$  acid at room temperature exclusively is merely a good quality vaseline. If incubations are carried out at  $38^{\circ}$  the mixture is hardened by melting 3 parts vaseline with 1 part of paraffin wax (M.P.  $55^{\circ}$ ) and allowing to cool. When using the  $N/1000$  and  $N/5000$  acids, it is advisable to use a fixative containing only a mixture of pure solid and liquid paraffin melted together and allowed to cool. The proportion may be as follows, 50 g. of solid paraffin (M.P. about  $49^{\circ}$ ) in 80 ml. liquid. This mixture may be varied in accordance with the prevailing laboratory temperature, and is not suitable for incubation.



## CHAPTER X

### SPECIAL FACTORS INFLUENCING THE RATE OF AMMONIA ABSORPTION

It is not necessary to know of these factors when carrying out the above determinations in accordance with the instructions; but it is essential when it is required to vary the quantities and conditions in the outer chamber. The influence of fluid volume in outer chamber, of temperature and other factors have been already discussed in Chapter II. Here the special factors of the *pH* value and saline content of the outer chamber arise for consideration.

**pH influence.** If for a given quantity of ammonium salt we consider the rate of ammonia absorption ( $A_1$ ) at high *pH* values and the rate ( $A_2$ ) at a *pH* where only a fraction exists as gas, the ratio of these two rates will be the ratio of the tensions of ammonia gas developed. This, however, is clearly the ratio of the degrees of dissociation of the ammonium ions, if we regard these for convenience as dissociating into ammonia gas and hydrogen ion, so that we have

$$A_1 = \alpha A_2. \quad (28)$$

Thus, when the ammonium ion is half dissociated,  $A_1$  is half the full absorption rate  $A_2$ . The degree of dissociation or the value of  $\alpha$  is given from the equation

$$pH = 9.4 + \log \frac{\alpha}{1 - \alpha}. \quad (29)$$

From this it will be seen that when  $\alpha = 0.5$ ,  $\log \frac{\alpha}{1 - \alpha} = \log 1.0 = 0$  and the *pH* of the solution is 9.4. At *pH* 9.4, therefore, the ammonia will be absorbed at half the full absorption rate (considering such absorption rates without any appreciable salt effect). Similarly at *pH* 8.4 it will be absorbed at 10% of the full absorption rate. Little advantage will be obtained in

itself by pushing the *pH* beyond 10.4, when 90% of the full absorption rate is occurring.

It may be noted that the *pH* of normal ammonia solution is approximately 11.6, 99.5% of the total ammonia being present as the free gas, and the *pH* of *N*/10 ammonia is approximately 11.1, with 98.5% as free gas. Such standard ammonia solutions containing the free ammonia may therefore be used in any investigations of absorption rates and various influences thereon, without the need of making standard ammonium salt solutions and adding alkali thereto.

**The effect of salt addition on the ammonia absorption.** Apart from the *pH* effect, the addition of certain salts to ammonia solutions can very greatly increase the tension of the ammonia gas therein, and therefore the absorption rate in the Units. Such effects of salt addition on ammonia tensions have been exhaustively investigated by Gaus (34) and by Abegg and Riesenfeld (18). The potassium salts were found to be very much more effective than the sodium, and amongst the salts of potassium, the carbonate and the metaborate have the most marked action. The effect of the cations goes in the following series: Mg—Ca—Li—NH<sub>4</sub>—Na—K, increasing from left to right, and the first three of these actually produce a *decrease* in ammonia tension. The effect of the anions goes in the following sequence:

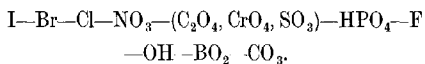


Table VI gives a comparison of the absorption rates at 38° C. in the Unit after the addition of various salts, 1 ml. of the saturated or standard salt solution being added to 1 ml. of a pure ammonia solution. The rate from pure ammonia solutions (2 ml. in outer chamber) is taken as the standard for column 4.

It will be seen that the absorption rate can be trebled by the addition of an equal volume of saturated potassium carbonate or metaborate to the solution in the outer chamber. Potassium fluoride is also very effective.

It may be taken that for every 1% saturation of potassium carbonate in the mixture there is an approximate increase of

4.4% in the absorption rate, and that at the same time for mixtures of equal concentration the time of clearance from the outer chamber is inversely proportional to the volume therein. From these data it will appear that a rough calculation of absorption times can be made for any concentration of potassium carbonate in the outer chamber and—within limits—for any volume of the mixed fluids.

Potassium metaborate is particularly effective for adding to rather concentrated acid solutions containing ammonia. Here carbonate would cause a marked evolution of carbon dioxide.

**The error of the ammonia determinations.** The possible lower limit of variable error in such determinations consists in the summation of that involved in the pipetting of 1 ml. acid into the central chamber and of the fluid into outer chamber, and the titration of the acid residue after absorption. A large number of such ammonia determinations were carried out with the Bang burette titrations before the horizontal burette was elaborated and gave a standard deviation or coefficient of variation of 0.5%. The results are given in Table VII, and exceed the possible lower limit of error.\* Titrations with the horizontal burette, and using the more refined pipetting already described, would give considerably reduced errors.

TABLE VI

<i>Substance investigated.</i>	<i>Concentration of substance in mixed fluid at 38°.</i>	<i>Absorption rate at 38° (<math>A_1</math> value, see equation 1 in text).</i>	<i>Calculated % increase in ammonia tension in outer chamber.</i>	<i>% increase in <math>NH_3</math> tension found by Abegg and Riesenfeld using 0.5N sols. of the substances in col. 1.</i>
KBO <sub>3</sub>	$\frac{1}{2}$ sat.	0.054	218	24
K <sub>2</sub> CO <sub>3</sub>	"	0.050	194	27
KF	"	0.045	165	19
K <sub>2</sub> HPO <sub>4</sub>	"	0.035	106	17
K <sub>2</sub> C <sub>2</sub> O <sub>4</sub>	"	0.024	41	15
KOH	20%	0.041	141	20
NaOH	"	0.037	118	13
NaOH	0.5N	0.019	12	13
Water	—	0.017	0	0
Glucose	25%	0.014	-17	—

\* 0.2-0.3% under the analytical conditions.

TABLE VII

<i>Quantity of ammonia-nitrogen added to outer chamber, mg.</i>	<i>Percentage 'recovered' by absorption in inner chamber.</i>	<i>Standard deviation of individual estimation as mg. ammonia-nitrogen.</i>	<i>Coefficient of variation of individual estimation.</i>	<i>Number of estimations.</i>
1.40	99.5	$7.0 \times 10^{-3}$	0.50	48
0.140	99.5	$6.7 \times 10^{-4}$	0.48	33
0.014	99.5	$1.0 \times 10^{-4}$	0.72	24

## CHAPTER XI

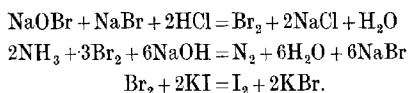
### OTHER METHODS FOR DETERMINING THE AB- SORBED AMMONIA IN THE MICRO-DIFFUSION PROCEDURE

**Bromometric.** Rappaport and Glaser (35) used such a method for exact blood urea determinations after distilling off the ammonia in a Parnas-Wagner apparatus (36). Following this Rappaport and Gutman (37) adapted their procedure to the micro-diffusion method subsequent to a reading of the paper by Gibbs and Kirk (23) on minute urea determinations with a reduced model of the Unit. They found it superior to the distillation method.

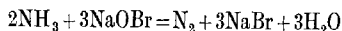
As used by Rappaport and Gutman, the ammonia is first absorbed by diffusion into 40% sulphuric acid in the detachable chamber of a Widmark alcohol apparatus. This is not nearly as efficient as the standard Unit, and it is unnecessary to use so strong an acid solution. After the absorption the chamber with acid is removed to a Hagedorn-Jensen tube in which it is neutralised, 5 c.c. of a hypobromite mixture added, and then a tiny crystal of KI and 2 c.c. of a hydrochloric acid solution made by diluting fuming hydrochloric acid with an equal volume of water. The free iodine formed is titrated with *N*/100 thiosulphate in the usual fashion with starch.

Since a very much weaker acid would have sufficed for the ammonia absorption, the subsequent and rather awkward neutralisation procedure described by them could be dispensed with and is not here given.

The essential reactions in the above method are (subsequent to absorption and neutralisation of the acid), according to Rappaport and Gutman :



The reaction between ammonia and hypobromites is, however, more usually given as



(e.g. Mellor, ref. 38).

Such a method would in no way increase the accuracy of the methods here described for solutions of Ranges I and II, and would only lengthen the procedure and the making of solutions. When using solutions of Range III it may possibly be of use, but owing to the necessary increase in volume and the use of many more reagents the writer would prefer the direct barium hydroxide titration with the Tashiro reagent for such minute ammonia absorptions.

The hypobromite solution used in the above method is made as follows :

- (a) *Bromine solution.* 20 g. KBr are dissolved in 100 c.c. of  $\text{H}_2\text{SO}_4$ , then 8 g. bromine (2.5 c.c.) are added, the solution shaken and made to mark with water. It keeps for a long time in the cold and stored in a brown bottle.
- (b) *Buffer solution.* 84.5 g. boric acid and 14.6 g. sodium hydroxide are dissolved in about 800 c.c. distilled water, boiled for 30 minutes to drive off any ammonia present and after cooling filled to the 100 c.c. mark.

Before the experiment, to 10 c.c. of the bromine solution in a 50 c.c. volumetric flask, dilute sodium hydroxide is added till the colour changes from brown to lemon yellow ; then about 25 c.c. of borate buffer are added and distilled water to the mark. This hypobromite solution should be made up fresh before using.

**Boric-sulphuric acid procedure.** Shortly before going to press a paper by I. Abelin (39) appeared in which the use of 2% boric acid to absorb the ammonia from the outer chamber was described, the ammonia being produced by the action of urease on blood urea. Methyl red and methylene blue indicator was used, the absorbed ammonia turning the indicator green, and the mixture was titrated back to a red end-point with  $N/100$  sulphuric acid. The author has subsequently investigated the method, but considers the end-point unsatisfactory when compared with the barium hydroxide titration. The

principle is certainly a good one, and if in practice not found particularly good in its present form, may very well prove advantageous by an alteration in the conditions or the reagents.

Some criticisms of the older method made by Abelin are not valid. He points out that there may be a loss of ammonia on titrating with alkali into the central chamber. This never happens in the actual practice of a titration, and can only arise with a large rush of the titrating alkali without stirring. A very large number of recoveries of ammonia in this and other laboratories have very amply proved that these are quantitative.

The practical difficulty of producing carbon dioxide free alkali no longer exists in the methods here described in which barium hydroxide solution can be made up in a few minutes from the stock commercial solution. That this solution deteriorates in the burette is not an objection in practice since it is always standardised by a titration against the acid; though admittedly it would be a greater convenience if the titrating fluid were also perfectly stable.

**Colorimetric or spectrophotometric.** After the ammonia absorption in dilute acid the procedure may be readily adapted to colorimetric or photometric examination. The titration method is much preferable on grounds of accuracy, and also of convenience when once the procedure has been going as a routine. For the minutest amounts (Range III), however, the spectrophotometric method may be preferred by some to the titration procedure, since it is not so dependent on a high grade cleaning of the Units.

The spectrophotometric procedure has in this way been used by Borsook. He used a Unit similar in dimensions to the standard design, and the phenate hypochlorite reagent as described by Van Slyke and Hiller (40), instead of the Nessler reagent.

In using these procedures, the problem is merely one of determining the ammonia in a pure weak acidic solution. The acid for absorption may be chosen as  $N/100$  or  $N/200$  sulphuric acid, without indicator. The Nessler reagent, if used, should not be directly added to the acid in the outer chamber, owing to possible loss of ammonia. It may be pipetted out and made

up with washings to 10 ml. in a small cylinder and 0.5 ml. Nessler reagent added, the reading with the Pulfrich photometer being then made with filter *S* 43, and using Figs. 14 and 15 in Krebs' description (41) of Cleghorn and Jendrassik's method for residual nitrogen in blood.

The extinction figure on the instrument is divided by the stratum depth used and the abscissa corresponding in mg. % residual nitrogen in these diagrams divided by 2 gives the gamma ammonia *N* in the 1 ml. of fluid examined.

For the very lowest ranges the central fluid may be 1.0 of pure acidic solution of *N*/1000 strength or less. 0.9 ml. is carefully pipetted out in the end into a small tube and 0.15 ml. Nessler added. With this mixed fluid the micro absorption tubes of 1 ml. capacity and 50 cm. stratum depth are filled and the extinction determined and compared with that for standard solutions similarly treated. In this way ammonia *N* down to 0.1 to 0.2  $\gamma$ /ml. should be determinable with an accuracy at about the 5% level. Borsook has in fact estimated 0.5  $\gamma$  with an error not greater than  $\pm 2\%$  (28).

Apart from such spectrophotometric determinations, ordinary colorimetry can of course be used in which the Nesslerised solution is directly compared with a similarly treated standard.

Recently Van Slyke and Hiller have revived the use of the blue colour produced by ammonia with an alkaline solution of phenol and hypochlorite for use in blood ammonia determinations.

For quantitative determinations, this was first used by Thomas (42, 43), who attributed its discovery to Berthelot.

Van Slyke considers it to be more delicate than the Nessler reagent.

#### Preparation and use of the Nessler and phenate-hypochlorite reagents.

*The Nessler reagent.* This may be prepared by grinding 10 g. mercuric iodide ( $\text{HgI}_2$ ) in a porcelain mortar with a little water, the mixture being rinsed into a dark bottle and the remainder of the water (100 ml. in all being used) in which are dissolved 5 g. potassium iodide and 20 g. pure  $\text{NaOH}$ , prepared from metal, added. The precipitate will settle completely within 24 hours, so that the perfectly clear solution can be



decanted off. The reagent should be kept in a well-stoppered dark bottle.

*The phenate-hypochlorite reagent.*

*Sodium phenate reagent.* This is made by dissolving 25 g. of phenol in a small quantity of water, adding 50 ml. of 40% NaOH and diluting to 100 ml. with ammonia-free water.

*The hypochlorite solution (Javel water),* containing 1 g. of Cl per 100 ml. Dissolve 50 g. of calcium hypochlorite, having 56.5% available Cl in about 500 c.c. of hot water. Mix this solution with one containing 50 g. of anhydrous potassium carbonate in 200 c.c. of cold water. Make the whole to a volume of 1 litre. Test the clear solution for excess calcium with  $K_2CO_3$  and add more of the latter if necessary to precipitate all the calcium. This reagent should be kept in the ice box in a number of small bottles, so that the chief source of supply is not opened each day, and so that there is a minimum of replacement of solution by air as the reagent is used. The active chlorine is estimated before use and at intervals of a few weeks as follows: To 5 c.c. of the Javel water are added 25 c.c. of water, 2.5 c.c. of 10% potassium iodide, and 2 c.c. of glacial acetic acid. The mixture is then titrated with 0.1N thio-sulphate which has been freshly standardised against 0.1N potassium bi-iodate solution.

In using the reagent, to each 5 c.c. of dilute acid containing the ammonia 1 c.c. of the sodium phenate solution is added and 0.5 c.c. of the Javel water. Mix, place in a boiling water bath for 3 minutes, cool to room temperature and compare with standard in a colorimeter.

**Gasometric.** As shown in the section on the bromometric method, nitrogen is liberated by the action of bromine on ammonia and in equivalent amounts. The nitrogen so liberated may be determined gasometrically.

## CHAPTER XII

### AMMONIA. BIOLOGICAL DETERMINATIONS

#### (1) Ammonia in urine.

0.5 to 1.0 g. of ammonia nitrogen are excreted per day by the normal human subject, but as high as 12 g. have been observed with diabetics. Ammonia must be formed by the kidney since, as shown by the author, none exists in the blood of the normal resting subject (5). The rate of its excretion is independent of the urine volume, but is closely related to the excretion of acid and also related to the total nitrogen output. This has been expressed in the following form by Raffin (44).

$$\frac{pH}{4} + \log(NH_3) - \log(\text{total N}) = K.$$

Hasselbalch (45) considers the urinary ammonia nitrogen expressed as a percentage of the total nitrogen at a pH of 5.8 ('reduced ammonia coefficient') to be a reliable method of measuring acidosis and the ability of the individual to combat acidosis. The percentage at pH of 5.8 varies from 2.2 to 5.5, but is much more constant for the individual.

*Principle of determination.* The ammonia concentration of urine being comparatively high (but going inversely as the rate at which urine is excreted by the kidneys), the solutions of Range I (page 76) are used. For accurate results some account must be taken of the urine rate in diluting the urine for the determination, unless 24 hours samples are being investigated, and it is even then advisable.

The following dilutions will be found suitable :

< 20 ml. per hour dilute	2 ml. to 100,
20- 50 ml. per hour dilute	5 ml. to 100
50-100 ml. „ „ „	10 „ 100
100-200 ml. „ „ „	20 „ 100
200-400 ml. „ „ „	40 „ 100
>400 use undiluted urine.	

These dilutions are easy to remember.

*Procedure.* The central chamber of the Unit is filled with 1 ml. acid of Range I, either from an Ostwald 1 ml. pipette, 2 ml. Bang burette with reservoir or simple pyrex tube pipette. Into the outer chamber is delivered 1 ml. of the diluted urine and the lid smeared with the fixative and placed in position. 1 ml. of saturated potassium carbonate is then introduced in the usual way. The Unit is left on the bench for 1.5 to 2.0 hours, the lid being then removed and the central chamber titrated with the alkaline solution of Range I. A blank determination is also carried out.

*Calculation.* The amount of the titrating fluid corresponding to the absorbed ammonia and reckoned as large divisions on the burette (each division = 0.01 ml.) is multiplied by 2 to give the  $\gamma N$  per ml. of the diluted urine. If  $a$  be the number of large divisions, then

$$\frac{2a \times 100 \times \text{dilution}}{1000} = \text{mg. } NH_3 - N \text{ per cent.}$$

$$\text{or} \quad \frac{a}{5} \times \text{dilution} = \quad \quad \quad \text{,,} \quad \quad \quad \text{,,} \quad \quad \quad \text{,,}$$

## (2) The Blood Ammonia.

*Principle and introduction.* The determination of the free ammonia of normal circulating blood has long been regarded as particularly difficult. The early literature, dating from the researches of Nencki and co-workers, has been reviewed by Parnas and Heller (46), Parnas and Klisiecki (47), and the subject has been recently reviewed by Schneller (48), in the *Ergebnisse*, and by Stanoyevitch (49).

Folin and Denis (50) were the first to find values under 0.1 gm. ammonia  $N/100$  ml. If the special conditions that affect

determination be not taken into consideration it is possible to obtain results as high as 2.0 mg./100 ml., and many published figures are of the order of 0.3 mg./100 ml. Table VIII gives a brief summary of the data published in recent years.

TABLE VIII

<i>Observers.</i>	<i>Date.</i>	<i>Human blood ammonia (Venous) γ ammonia N/100 ml.</i>	<i>Ref. No.</i>
Parnas - - -	1925	30	54
Van Slyke <i>et al.</i> - - -	1925-26	0-100	55
Klisiecki - - -	1926	26-15	56
Labbé	1929	30-72	57
Nepveu and Hejda		mean 47	
Stanoyevitch - - -	1931	19-42 } men mean 26 } 17-30 } women mean 23 }	58
Folin - - -	1932	60-116 (after 30 mins.) 75-171 (after 1 hour)	59
Fuld - - -	1933	28-60 mean 42	60
Kalk and Bonis - - -	1933	10-45	61
Conway; Conway and Cooke	1935	0 (Immediate) 44 (mean, 5 mins. after shedding) 70 (mean, 60 mins. after shedding)	62 & 51

It will be seen that with the micro-diffusion method there would appear to be no free ammonia in normal circulating human blood. In this method all aeration and distillation are avoided. Numbers of determinations can be rapidly carried out so that the true curve of ammonia formation from zero time of shedding can be readily obtained. From zero up to five minutes after shedding (see Fig. 22), the ammonia content rises in a steep curve up to a mean of 46γ ammonia N/100 ml. (written as 0.46 'gammils' N in the suggested notation). From five minutes onwards there is a slow formation of ammonia (Fig. 23), representing at room temperature (16°) about 0.43γ N/100 ml. blood per minute. The rate of for-

mation is considerably affected by temperature, being  $0.97\gamma$   $N/100$  ml. per minute at  $24^{\circ}$ . At  $38^{\circ}$  the formation shows a curious phasic appearance (51, 52), and this phasic formation occurs for the rabbit's blood at room temperature, the rate of formation being here much more rapid than for man.

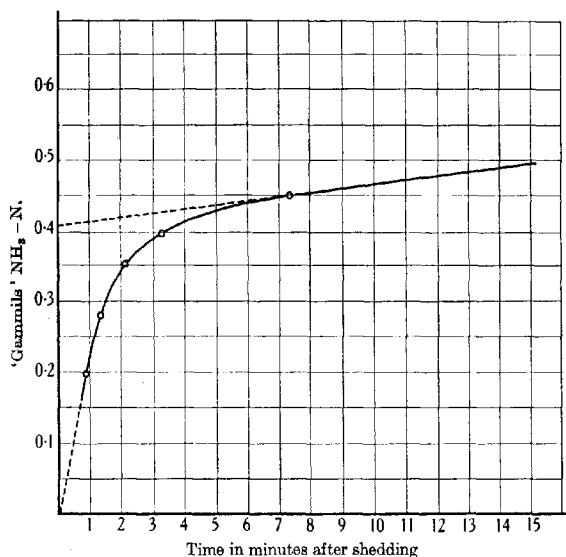


FIG. 22. The curve is constructed from the means of a number of determinations on 12 blood samples (about 25 ml. each) from seven different subjects. The blood was collected in each case direct from an arm vein through a hypodermic needle into an open Erlenmeyer flask lined with solid paraffin. Mean room temperature  $16^{\circ}$ . This curve, as well as those in Figs. 23 and 24, is drawn at a somewhat higher level than the original (*Biochem. J.*, 29, 2755), owing to a more extended investigation of the rate of ammonia emission from a blood-carbonate mixture.

The phasic formation appears generally characteristic as it is evident also in fowl's blood.

That preformed ammonia is present in measurable amounts after exercise and in certain diseased conditions is very likely, but has not been investigated so far by the Unit technique. Whatever stage of formation it is desired to investigate or whatever conditions with regard to their influence on the preformed

ammonia, the procedure here described will be found suitable, with slight alterations according to the conditions.

Referring briefly to other methods of blood ammonia determinations, it may be said that these are either developments of the original Folin technique (e.g. Nash and Benedict (53), using special absorbers) or a steam distillation method elaborated

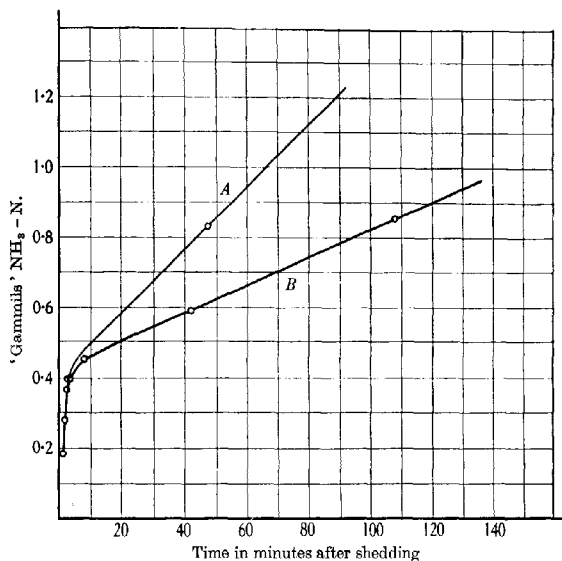


FIG. 23. Curve B is the same as Fig. 22 on a ten times smaller scale. Curve A represents the ammonia formation in blood maintained at 22°, from two subjects. (*Biochem. J.*, **29**, 2755.)

by Parnas and Wagner (36). The ammonia for 5 ml. blood—or 2 ml. as with the Parnas-Wagner method—is finally present in about 4 or 5 ml. fluid. It is then colorimetrically determined after adding Nessler's reagent. Considerable differences exist in the reports of the delicacy of this reagent for such a purpose. Van Slyke and Hiller (40) state that 0.001 mg. ammonia nitrogen (the ammonia from 5 ml. blood containing 0.020 mg. ammonia N/100 ml.) in 5 ml. fluid is not visible at all with the Nessler reagent. Parnas and Heller, on the other hand (46),

claim to be able to detect the difference between 0.001 and 0.0009 mg. ammonia *N* in 5 ml. (corresponding to the ammonia from 2 ml. blood containing 0.050 mg./100 ml.). This latter would in any case seem quite workable in general with the spectrophotometer.

Van Slyke and Hiller (40) make use now of the phenate hypochlorite reagent as already described.

In the method to be described (*following the general ammonia determinations of Range III*), using the hydrochloric-barium hydroxide procedure, the ammonia is finally present in 0.7 ml. of a 0.0002*N* hydrochloric acid solution in which is incorporated the indicator as described. The titration error here sets the limit of delicacy for the determination since no appreciable error is introduced by the ammonia absorption itself. The titration error of 0.7 ml. *N*/5000 acid (with indicator, see below) with 0.9 ml. final fluid corresponds to 0.02% ammonia *N* as a standard deviation and for 1 ml. of original fluid investigated is equivalent to 0.002 mg./100 ml. When blood is used and the absorption time is suspended the error is 0.003–0.004 mg./100 ml. This is approximately five times more delicate than the method of Van Slyke and Hiller (40) and permits the same accuracy using only 1 ml. blood or 0.5 ml.—as will appear—instead of five.

In the ordinary use of the Unit the absorption at room temperature takes two hours under such conditions, which is too long a period for blood ammonia determinations as there is a steady formation of ammonia in the presence of the alkali. *When we consider, however, that each Unit is turned out from the same mould with the same dimensions and surface relations, and further, that since after the first few seconds the fluid surfaces are perfectly still, a uniform passage of ammonia may be expected and in fact occurs. An error of less than 5% is introduced by cutting short the absorption to about the half period, but less than 1–2% when allowed to go to 83% absorption (as with 0.5 ml. blood and 0.5 ml. saturated carbonate for 20 minutes).* Whatever accuracy may be lost here is more than compensated for in comparison with other methods by the entire freedom from contamination during the absorption period. In 10 minutes approximately 42% of the

ammonia is absorbed at room temperature with 1 ml. blood plus 1 ml. carbonate and 83% with 0.5 ml. blood and 0.5 ml. carbonate. The ammonia formation in the presence of the alkali over this period is comparatively small, and can be accurately allowed for.

*Procedure.*

*Preparation of Units.* The whole success of the method depends on the Units being properly cleaned in the manner already described. The reagents of Range III (Table V) are used with this method and the number of Units required are prepared up to the point of introducing the blood into the outer chamber and left aside for about twenty minutes before using.

The saturated carbonate may be introduced before or after the introduction of the blood into the outer chamber, depending on the speed with which the determinations are required after shedding. If required immediately after the blood is shed it is convenient to have the Units prepared with all additions—acid and carbonate and lid in position—thus allowing for a very rapid blood determination. If the determination is not required until five or ten minutes have elapsed after shedding, it is better to add the blood first. Allowing twenty minutes after the Units are prepared and before blood introduction will ensure the absorption of small traces of ammonia that may possibly exist in carbonate and fixative.

To obtain the maximum possible accuracy, and to ensure completely that no contamination of any kind will interfere during the absorption period, a number of Units may be prepared with every addition except the blood and left aside for twenty minutes or half an hour. At the end of this period the lids are detached in succession, the central acid being titrated to neutrality, the mixture being then sucked out—using a filter pump and pyrex tube—a refill of acid made, and the lid replaced.

The acid is introduced as 0.7 ml. from a pyrex tube pipette. This amount will be found to cover the surface of the internal chamber, when the Units have been carefully cleaned.

*Collection and introduction of the blood.* The manner of collection depends on the object of the determination. When



human venous blood from the normal resting subject is received into alveolar air there is no apparent free ammonia (Fig. 24).

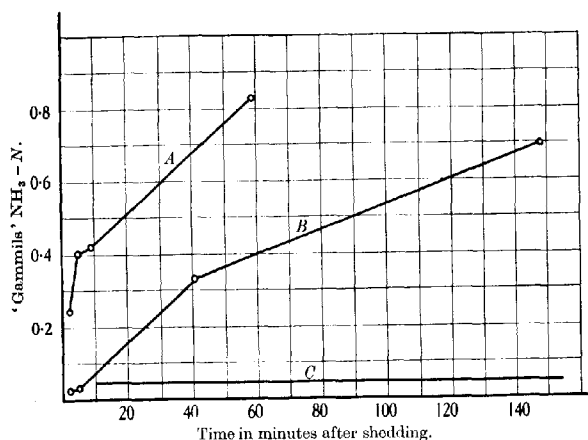


FIG. 24. Curve *A* represents the blood ammonia formation for two subjects, the blood being collected in an open Erlenmeyer flask. Curve *B* gives the formation of ammonia in blood from the same two subjects collected in alveolar air. Curve *C* represents the blood ammonia formation after collection in pure carbon dioxide at atmospheric pressure for one subject, the curve representing the mean line through 11 determinations made up to 128 mins. after shedding (*Biochem. J.*, **29**, 2755).

If the blood is allowed to drop through the air into a container ammonia forms, as already described, to reach in about five minutes (Figs. 22, 23) a level of 0.46 'gammil'  $\text{NH}_3 - \text{N}$ , rising then slowly from this point onwards.

If the ammonia content after five minutes from shedding is required—and this will correspond to the other methods at present in use—a few ml. of blood are taken from an arm vein in a syringe specially cleaned, and then this blood is introduced into a small tube or flask with a little ammonia-free potassium oxalate.

When a large number of determinations are in prospect on the blood sample and 10–25 ml. are required, the blood may be allowed to flow directly from the vein through a hypodermic needle (the syringe being removed) into a small flask containing a little oxalate. A rubber tourniquet tied around the arm above

the elbow will assist in distending the veins and securing the necessary pressure, the tourniquet being immediately loosened when enough is taken and the arm flexed on a piece of cotton wool soaked in alcohol and pressed on the site of the puncture.

If the blood is being received into alveolar air, a tonometer of about 200 ml. capacity or larger and containing a little oxalate is filled with alveolar air, and the hypodermic needle to be introduced is attached to a long tube (best coated internally with paraffin), the syringe not being used here for assistance in entering the vein. The first drops of blood may be discarded and then the tube inserted down into the tonometer, the stopper of which has been removed immediately before.

*Introduction of the blood after collecting, etc.* The Units being previously completely prepared, with the carbonate already present in the outer chamber, 1 ml. of the blood is pipetted therein, the time being noted and the contents mixed by rotation 15 times in about as many seconds. After allowing the absorption to proceed for ten minutes the lid is removed and the content of inner chamber titrated. At least one blank Unit is titrated and one control set up in a similar way. The control may suitably consist of 1 ml. of standard solution containing 2 'gammils'  $\text{NH}_3-N$ .

The titration is carried out in the ordinary way, using a fine pyrex glass rod. A colour change will be evident at the end-point with an addition corresponding to 2 or at most 3 small divisions. Accuracy in determining the end-point will be increased with a little experience. A very faint green appearing in an almost colourless solution when titrating with the barium hydroxide solution is preferred by the writer as the end-point to be aimed at.

#### Solutions required.

The absorption acid and titrating alkali are those of Range III (Table V, p. 76, under general ammonia method).

The only additional material required is:

*Oxalate (ammonia-free).* About 50 ml. of a strong solution of potassium oxalate are evaporated in an open pyrex dish after bringing to a pH of 10 or 11 by adding a little alkali, the reaction being tested by a universal indicator. The water volume is maintained by occasional addition. The mixture is kept boiling for about 10 minutes and then

evaporated until the solid separates; after cooling, the crystals are filtered off on a small Buchner funnel and preserved in a dessicator.

*The fixative.* The same fixative is used here as for chloride determinations (4): 40-50 g. paraffin (M.P. about 40°) are melted with 80 ml. of pure liquid paraffin and cooled. Vaseline should not be used, as it contains traces of ammonia.

2 'gammils'  $\text{NH}_3$ -N control solution. 0.471 g. of pure  $(\text{NH}_4)_2\text{SO}_4$  is dissolved and made up to a litre with distilled water. 2 ml. of this stock is diluted to 100 ml.

TABLE IX

Absorption time. Mins.	Ratio of ammonia absorbed from water- carbonate to that from blood- carbonate mixture.	The absorption as an approx. percentage of the total ammonia in the aqueous solution. %	Allowance in the final result for the special deaminating action of the alkali. $\gamma$ ammonia N/100 ml. blood at				
			25°	20°	15°	10°	0° C.
10	1.15	52	11	7	4	3	1
12	1.14	57	14	8	5	3	1
14	1.12	61.5	16	10	6	4	2
16	1.11	65.5	19	12	7	5	2
18	1.11	69	22	14	9	5	2
20	1.11	72	25	16	10	6	2

In the above table made out for the pyrex Unit as made by Messrs. A. Gallenkamp, London, it is understood that 1 ml. blood or water is added to 1 ml. of saturated potassium carbonate in the outer chamber from pipettes exactly calibrated for this purpose, and that the mixing is carried out with a fixed routine of fifteen rotations in approximately 15 seconds. The allowances in the fourth column for the special deaminating action of the alkali have a temperature coefficient of 10% per degree (From the *Biochem. J.*)

*Calculation of the blood ammonia.* This is based on the result of the control ammonia. The number of large divisions on the burette corresponding to the blood ammonia is divided by the number for the control and the result multiplied by 2.0. This must at the same time be multiplied also by 1.15 (where ten minutes are allowed for the absorption) owing to the fact that the ammonia comes over somewhat more rapidly from a water-carbonate mixture than from a blood-carbonate mixture. (These rates were at first considered to be the same but a fuller investigation under improved conditions gave the factors shown in Table IX.)

An allowance must also be made for the ammonia formed by

the action of the alkali on the blood and absorbed in the given time. Such allowances are given in Table IX, and are listed for different room temperatures. An example will show clearly how the calculation is made.

EXAMPLE. (All titrations carried out in duplicate, and the control solution containing 2 'gammils'  $\text{NH}_3 - \text{N}$ .)

Titration of 'blank' unit	-	-	-	35.6 large divs.
Titration of 'analytical' unit	-	-	-	31.3 " "
Titration of 'control' unit	-	-	-	14.2 " "
Time of absorption	-	-	-	10 minutes.
Room temperature	-	-	-	15° C.

The amount of the absorption in the analytical unit corresponds to 35.6 - 31.3 or to 4.3, and in the control of 21.4 large divisions (each large division of the burette corresponds to 0.01 ml. of the solution of Range III).

Calculation:  $\frac{4.3}{21.4} \times 1.15 \times 2.0 = 0.462$  'gammils'  $\text{NH}_3 - \text{N}$ . The factor

derives from Table IX and 2.0 represents the control.

From the gross value of 0.462 'gammils'  $\text{NH}_3 - \text{N}$  0.04 is subtracted to allow for the special deaminating action of the carbonate as given in Table IX, column 4.

Before titrating with the barium hydroxide it is advisable to run out about 7-10 ml. from the burette, and also to have the cork of the reservoir bottle and of the soda-lime guard painted and sealed with melted paraffin. It will be seen that in the use of the method the exact strength of the barium hydroxide is of no consequence.

(3) **Determination of the total ammonia formed after 24 hours in sterile blood at room temperature or after some hours action at 38°.** After 24 hours at room temperature the amount in human blood is approximately 0.7 mg.  $\text{N}/100$  ml., deriving mainly from the breakdown of adenylypyrophosphoric acid (it is approximately 1.8 mg.  $\text{N}/100$  ml. in rabbit's blood). The ammonia formed by the action of the alkali may be neglected. Using the method of suspended absorption, the mean of a duplicate determination may be relied on to within 2-3% error. Much smaller volumes of the blood may be used with 1 ml. of saturated carbonate and enough time allowed for the total absorption. Solutions of Range II would then be appropriate.

A suitable procedure here would consist in using 0.2 ml. of the blood and 1 ml. saturated carbonate and allowing the absorption to proceed for 45 minutes, in which time with these volumes the absorption is complete. Solutions of Range II could be used with respect to the acid and alkali.

Alternatively 0.1 ml. blood and 1 ml. carbonate may be used with solutions of Range III. (*Vide* p. 76 and p. 79.)

#### (4) The preformed ammonia in muscle.

From the moment of its excision from the animal ammonia begins to form in muscle, and if the muscle be ground with water or saline the rapidity of the formation is enormously accelerated, passing from an apparent value of approximately 7 to 40-60 'gammils'  $\text{NH}_3$ -N within five minutes. Its ammonia content also increases during activity as is now well known from the work of Embden (63, 64) and, later, of Parnas (65). As it is necessary to have the muscle finely divided, chemical or physical measures must be taken to prevent the formation during this division. In the method of Embden (66), who discovered adenylic acid in muscle (now known to be combined with phosphoric acid) and showed it to be at least the chief mother substance of the formed ammonia, the muscle is firstly frozen by immersion in liquid air contained in a Dewar flask, the muscle being held under the surface of the liquid by a forceps to ensure rapid freezing, after which it is ground in a fold of linen or muslin in a mortar—which may in turn be cooled with some liquid air.

The powder is then introduced into weighed glass-stoppered vessels containing a measured amount of 4 or 1% hydrochloric acid (somewhat modified in later experiments). This fluid is considered to stop any further ammonia formation, prior to the ammonia determination.

The ammonia method used by Embden is a modification of the old method of Nencki and Zaleski, in which distillation was carried out *in vacuo* after the addition of excess magnesium oxide at a temperature of 30° (immersion in water bath). In the modification ground glass joints are used and the distillate (with air current sufficient to produce a positive

pressure of about 11 mm.) received into 5 ml. or so of standard acid containing some Tashiro's reagent. The distillation procedure is continued for 35 minutes. It may also be noted that the magnesium oxide is added as 15 ml. of a suspension containing 2 g. of the light magnesium oxide.

The method of Embden may now be considered as chiefly of historical interest.

In the method of Parnas (36, 46, 67, 68, 69), instead of freezing in liquid air the tissue is rubbed with a cold saturated borax solution. In the procedure is used a rough, deep glass mortar and a very large pestle. At the bottom of the mortar is a little quartz sand, sharp and granulated, and a few c.c. of cold saturated borax solution, previously boiled and ammonia-free. The muscle is thrown in and squashed as quickly as possible with a strong, pressing and rubbing motion. Experience must be acquired, but offers no difficulty. The method is suitable only for small muscles. Note that the ammonia content in grinding in the ordinary way passes to 4 to 6 mg. % and with the borax solution one finds 0.4 to 1.0 mg. %, mostly 0.6 to 0.7 mg. %. Similar results are got after freezing in liquid air and then proceeding to the borax grinding. The subsequent ammonia is then determined by the well-known Parnas method as modified in later adaptations.

**Micro-Diffusion method for determining the preformed tissue ammonia.**

*Preparation of Units, etc.* These are prepared as for determining the ammonia content of blood, the solution of the Range III, Table V, being used in the burette. The saturated carbonate may be introduced beforehand into the outer chamber or added after the introduction of the muscle-phosphate mixture. As with the blood ammonia, where the highest accuracy is required the Units may be firstly prepared, then the central chamber titrated after 30 minutes and the contents sucked out, being replaced by a fresh acid delivery. Such a procedure, however, is unnecessary if the fixative is absolutely free of ammonia and if the Units have been carefully cleaned.

*Preparation of tissue suspension and ammonia determination.* Here use is made of the fact first discovered by Parnas

*et al.* (70, 71), that phosphate suspends the ammonia formation in muscle extracts for upwards of half an hour, but the method is also applicable, with suitable modification, to tissues prepared by freezing in liquid air. Taking the gastrocnemius of the *Rana temporaria*, for example, this is excised immediately after the killing of the animal or subsequent to whatever conditions have been allowed to act on it. It is quickly weighed on a tared watch glass and introduced into 2.0 ml. of  $M/10$  phosphate and some fine quartz sand (Merck's purest) in a small clean mortar. The phosphate solution is formed by mixing equal volumes of  $M/10$  alkaline and acid phosphate. The pH of this mixture is approximately 6.8. Immediately after introducing the muscle into the phosphate it is ground with as large a pestle as convenient, the grinding being conducted as rapidly and efficiently as possible. When the muscle is thoroughly ground the mixture is poured into a small clean tube and allowed to settle for about one minute, when duplicate 0.5 ml. volumes, either directly or after clearing by centrifuging, are pipetted off into the outer chamber of prepared Units already containing 1 ml. saturated potassium carbonate (though this may be added subsequently).

The Units are left aside on bench after mixing contents of outer chamber, for 1 hour and then titrated. *Alternatively, if a quick result is desired, the absorption is suspended after 20 minutes, when 93% of the ammonia will have come over.* The suspension will here occur at a region of the absorption curve when the change with time is small.

*Possibilities of the method with regard to ammonia absorption and titration.* Concerning the ammonia absorption, it is clear from accounts of previous determinations that this offers no difficulty, but proceeds automatically and after 1 hour is complete under the conditions (93% after 20 minutes).

The titration possibilities are such that  $1\gamma$  ammonia  $N$  can be determined to a standard deviation of 0.02.  $1\gamma$  ammonia  $N$  corresponds to about 20 large divisions of Range III solution on burette and is contained in about 0.14 g. resting muscle.

If 0.5 ml. extract is pipetted into outer chamber and contains  $1\gamma$  ammonia  $N$ , this means a dilution of about 1 in 3.5.

The dilution in the above procedure will be about this order—usually somewhat greater.

*Results obtained for muscle with the above method.* The average of 25 determinations, using phosphate as above, was 0.72 'gammil'  $\text{NH}_3$ -N with a standard deviation of 0.05. The mean of a large number of determinations by Parnas and co-workers was 0.85 mg. %  $\text{NH}_3$ -N, but the best values were placed by him between 0.5 and 0.7 mg., and the true value as probably lying within 0.5 and 0.6. There was no significant difference found by him using cold saturated borax solution and a previous freezing in liquid air.

(5) **Determination of the total ammonia formation in muscle.** (See subsequently the adenylypyrophosphate determination.) This follows simply from the previous description, the ammonia content of the muscle here being upwards of ten times greater. A considerably greater dilution of the muscle suspension may be used to keep within Range III or the same dilution with Range II solutions, the latter giving more accurate results.

We can proceed in a variety of ways, remembering that each g. of muscle will give about 50 $\gamma$  ammonia N. Thus with a 1/5 extract 0.5 ml. in the outer chamber will give 5 $\gamma$  or 16.7 large divisions with solutions of Range II. With 0.5 ml. in the outer chamber with 1 ml. carbonate, 1 hour may be given for complete absorption at room temperature.

The absorption rate of ammonia from muscle extract is the same as from water.

TABLE X

Water.	Ammonia content. 'gammil' N.	References.
Rain water -	0.3 - 4.2	Boussingault (72); Smith (73).
River water -	0.01 - 4.0	Boussingault (74).
	0.58 - 1.9	Knop (75).
Sea water -	0.13 - 0.9	Audoynaud (76); Dieulafait (77);
	(11.4 observed in Adriatic)	Thorpe and Morton (78);
Spring water -	Generally lower than river or sea water	Vierthaler (79). Mellor (80).



(6) **Ammonia content of natural waters** (e.g. water from rivers, springs, etc.). Table X gives amount in different kinds of natural water, and the present method may be used when a titration procedure is preferred to Nesslerisation.

For these ammonia determinations the solutions for the third or lowest Range are used, each large division on the burette being equivalent to 0.050 $\gamma$  ammonia *N*, or when 1 ml. of the water is being used for the analyses to 0.05 'gammils'. Blank determinations are carried out at the same time.

Into the outer chamber is introduced 1 ml. of the natural water, mixed and allowed to stand 2 hours at room temperature. If a rapid result is desired the absorption is cut short after 10 minutes by removing the lid and titrating, the ammonia found being then multiplied by 1.95 at 18° C., with a decrease of 2% in factor for every degree increase in temperature. This latter procedure involves at most a 5% error in the ammonia, which for the minute quantities in this determination may be considered negligible.

*Calculation.* Each large division on burette is equivalent to 0.05 $\gamma$  ammonia *N*. This value will need to be corrected by a factor found by titrating one or two acid deliveries. When 1 ml. of the water is used each large burette division represents 0.05 'gammil,' mg. per litre or part per million.

*Solutions.* Range III solutions (Table V, p. 76).

## CHAPTER XIII

### TOTAL NITROGEN

WHEN large numbers of determinations of total nitrogen are being carried out either of a macro or micro kind, the micro-diffusion principle is of value in considerably diminishing the work in such serial determinations, and also for all micro ranges increasing the accuracy of the procedure (e.g. Bentley and Kirk (9)).

The volumetric accuracy alone need not fall below that for the full macro determination, as shown in Part III, and it is evident at the same time that the elimination of the usual distillation removes an important source of error. With quantities as low as 5γ ammonia nitrogen, Kirk and Bentley obtained an error of only 0.7% (standard deviation) for the full volumetric procedure, and of 1.2% for the complete process with thiobarbituric acid, *p*-amino-benzoic acid and *p*-brom-acetanilide in amounts giving from 3 to 10γ ammonia N.

The use of certain catalysts in excess should be avoided—for example, copper sulphate, since it slows the absorption considerably when in comparatively high concentration. It may still be used in small quantities of 0.1% solution. Copper is not so good a catalyst as tellurium, selenium, etc., as shown by Osborn and Wilkie (87) in an extensive analysis of the catalytic action of various metals, etc., with respect to Kjeldahl incinerations. In this way they found that the safest and best catalysts in order of efficacy were mercury, tellurium, titanium, iron and copper. For Unit determinations tellurium would seem one of the most suitable, but a combination of 0.1% copper sulphate and Superoxol (or Perhydrol), as used by Bentley and Kirk, gives excellent results, and has the merit of being fully tried out.

For the alkalisation of the digest or aliquot part of the

digest saturated potassium metaborate would appear to be most suitable and may be stored at about 40°C, with solid substance in evidence.

Saturated potassium metaborate and saturated potassium carbonate have practically the same effect in raising the ammonia tension of solutions, and hence increasing the absorption rate, but the carbonate cannot be used here owing to the high acidity of the digest.

The following account gives examples of incinerations and ammonia determinations at various levels, but they may of course be suitably changed for special conditions.

#### Macro-Kjeldahl determinations.

##### EXAMPLE 1. *Wheaten flour.*

*Incineration.* 0.8 g. of the flour is weighed into a 300 ml. Kjeldahl incinerating flask. 10 ml. of pure sulphuric acid are added and approximately 6 g. of sodium sulphate. Then about 0.15 g. powdered tellurium (procurable from Dr. T. Schuchardt, Görlitz), or other suitable catalyst, added and the mixture incinerated in the usual way with a small funnel in the top of the incinerating flask, and once and a half the clearing time of the fluid being allowed, which will mean usually about 20–30 minutes, though longer may be required.

*Ammonia determinations.* Subsequently the mixture is cooled, 50 ml. water added cautiously and cooled under the tap. The mixture is now made up to 200 ml. in a volumetric flask and 0.5 ml. in duplicate is pipetted into the outer chamber of two Units. The pipetting should be done with a simple straight tube pipette taking 20 seconds to deliver, and the final fluid blown out immediately after delivery with the tip touching the glass (see Chapter V).

The central chamber of the Units contains acid of Range I. 1 ml. of saturated potassium metaborate is introduced in the usual way after placing the lid with fixative in position, and the contents of the outer chamber mixed by rotation and left on the bench for 1 to 1½ hours or incubated at 38° for about 50 minutes.

*Titration and calculation.* The Units are titrated with the alkali of Range I. Each large division on the burette corresponds to 0.1% N in the original flour, or similar substance analysed in the above manner.

Owing to the very small content of ammonia in tap water as a rule, this may be used for the dilutions, a blank value being also run. The solutions are described on pp. 76 and 79.

**EXAMPLE 2. Urine.**

*Incineration.* 2 ml. of urine are pipetted into the incinerating flask from a straight tube pipette.\* The pipette, about 15 to 20 cm. long, should take about 20 seconds to deliver, the final amount being blown out gently with the tip against glass and rotating while blowing. Remaining procedure as in Ex. 1.

*Ammonia determinations, etc.* After the incineration as above the dilution is made to 250 ml. and the ammonia determination made in the same way. The number of large divisions neutralised by the absorbed ammonia divided by 20 gives the percentage nitrogen in the original urine. If the urine is dilute more should be used in the incineration. As a rough guide 1 ml. may be used up to 100 ml. per hour urine, but at greater rates the volume incinerated should be increased in proportion to the urine rate.

*Accuracy of the above macro-Kjeldahl determinations.* The accuracy of such procedures on the purely volumetric (including titration) side is analysed in Part III. It is shown there that *the diffusion technique can give an accuracy as great as the full macro-Kjeldahl conducted on the 25 to 50 ml. titration level.* With regard to the passage of the ammonia from the Kjeldahl digest to the acid, it is obvious that the diffusion procedure is much less liable to error, and goes automatically without any observation being required during the process.

**Solutions, etc., required for the above determinations.**

Pure sulphuric acid.

Solutions of Range I for ammonia determination (Table V, p. 76).

Tellurium powder.

Sodium sulphate.

Saturated potassium metaborate.

\* A standard 2 ml. pipette is also suitable.

**Micro-Kjeldahl determinations.**

The above determinations were described as macro, though usually somewhat more material is taken in the typical macro procedure. The *N* incinerated was above (as a rule) 1.0 milli-equivalent. In the following the determination of total nitrogen, using about 1/250 of this amount and about 1/2000 respectively in the incinerations is described, maintaining even at this lowest level, a range of accuracy of the 1 to 2% order.

**EXAMPLE 1.** *Non-protein nitrogen in blood—tungstate filtrate* (following the method of Cleghorn and Jendrassik for the incineration, ref. 82.)

*De-proteinising.* This is carried out by the method of Folin and Wu (83). 1 part of blood is haemolysed in 7 parts of water and then treated successively with 1 part of 10% sodium tungstate and 1 part 2/3*N* sulphuric acid. The mixture is vigorously shaken and allowed to stand until it has assumed a chocolate colour (about 15–20 minutes). The mixture is filtered or centrifuged.

*Incineration.* 2.0 ml. of the clear Folin-Wu filtrate, 0.2 ml. of 40% sulphuric acid and 0.2 ml. of Perhydrol (Merck's) are introduced into a pyrex or Jena glass test-tube. The tube is heated under the fume cupboard directly over a micro-burner, and the contents concentrated by boiling until dense anhydride fumes appear. The heating is continued for a further two minutes, using a smaller flame, the point of which does not touch the tube.

*Ammonia determinations.* After cooling, the contents of the tube are carefully washed out with three successive portions of 0.3 ml. distilled water, the fluid being transferred by a fine tube pipette (about 2 mm. internal diameter) to the outer chamber of a Unit. Each time the fluid should be removed as far as possible. It is important for the subsequent absorption that the fluid volume should be fairly accurate, a marked increase in volume appreciably delaying absorption as already shown in an earlier chapter.

Range I solutions, p. 76, are used for the ammonia deter-

mination, and after introducing the acid and preparing the Unit as usual, 1 ml. of saturated potassium metaborate is introduced into the outer chamber, the contents mixed as already described and the Unit incubated at 38° for one and a half hours or left aside on the bench for two hours at usual room temperature.

It is an advantage if the saturated potassium metaborate has been previously stored at a temperature of about 40° in the incubator.

*Calculation.* Each large division on the burette corresponds to 2γ ammonia N, and each incineration to 0.2 ml. blood. Therefore each large division corresponds to mg. N/100 ml. blood, the results being corrected for any alkali factor if present. The determination should be carried out with usual blank and occasional controls and corrections made for alkali and pipette factors.

*EXAMPLE 2. Total nitrogen determination at the 5γ level of N.*

**Kirk's method.** In this method, one of a series of 'drop scale' analytical methods, after the initial incineration of a pure compound contained in 20-40 c.mm., the ammonia is determined by the diffusion principle. In applying this, Bentley and Kirk (9) have designed another type of diffusion vessel which they consider somewhat better suited than the standard Unit for the ammonia recovery from small Kjeldahl digests, which are necessarily diluted. Since, however, the final volume of incinerated fluid plus washings used in this way are only 1.50 ml. in Kirk's method, the writer believes that the standard apparatus could be used to give similar results (though Bentley and Kirk's design may in addition prove very useful for other micro-determinations, where an upper surface or roof comparatively free from fixative is desirable). Using 0.5 ml. of saturated potassium metaborate as the alkali with 1.5 ml. of digest and washings in the standard Unit would give an absorption time of about 2 hours at 38°, or about 3 hours at room temperature. There is, therefore, scarcely appreciable saving in time since Bentley and Kirk used a 2 hours absorption period. 5γN will be equivalent to 16.7 large

divisions with the solution of Range II. Since, however, the results obtained with the whole procedure, which include the capillary pipette measurements of 20 c.mm., are so striking, the description of it is included here. An accuracy of 1-2% is reached with the 5 $\gamma$  level of total nitrogen determination, which includes measurement of volumes of the order of 20-40 c.mm. with capillary pipettes.

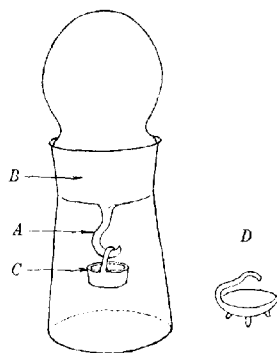


FIG. 25. The Kirk diffusion cell for minute total nitrogen determinations (from *Mikrochemie*, 21, p. 261). *Vide text.*

*Apparatus.* The diffusion cell used by Bentley and Kirk is illustrated in Fig. 25. The total height of the little flask was 45 mm., the ground stopper of glass (B), 25 mm. in diameter. The ground stopper carried a hook on the bottom from which hung a cup C, 12 mm. in diameter and about 5 mm. in depth. A second cup D, constructed in a similar way, was provided in addition with three short glass legs 3

mm. long. The digestion vessel consisted of a bulb, 13 mm. in diameter, blown on the end of 70 mm. of tubing, 7 mm. in diameter.

The remainder of the equipment consisted of the capillary pipettes and burette required (the latter with titration table, see Chapter VII, Part I), and a micro glass burner for digestion (described by P. L. Kirk, 84).

#### The solutions used.

1. Concentrated sulphuric acid, which has been redistilled in glass.
2. Dilute copper sulphate, made by dissolving the purest copper sulphate in water to about a 0.1% solution. Other catalysts, such as mercury or selenium salts, may be used in corresponding quantities.
3. Superoxol or Perhydrol, diluted with 2 volumes of water before use.
4. Strong caustic solution, either 50% or saturated solution of sodium hydroxide.

5. Bentley and Kirk also use  $N/40$  sulphuric acid for absorbing the ammonia—enough to form about 100% in excess—and  $N/40$  NaOH to titrate from a capillary burette.

In the use of the present standard Unit these would be replaced by solutions of Range II.

*Procedure.* (From the description of Bentley and Kirk, 9.) The sample, containing from 1 to 10% of nitrogen, is accurately measured with a capillary pipette into the digestion flask. To this are added 0.1 ml. of the strong sulphuric acid and a volume of copper sulphate solution approximately the same as the sample volume. The digestion is carried out by heating over a micro glass burner until charring is complete, after which the digest is clarified by addition of about 30 c.mm. of the dilute Superoxol. Heating is continued for about 2 minutes after the digest becomes clear.

A wad of cotton, wound on the end of a small glass rod, is dipped into the strong caustic and wiped around the interior of a clean, dry, diffusion flask (Fig. 25), about 1 cm. above the bottom. This forms a ring, past which no sulphuric acid can creep without being neutralised. The digest is then transferred with rinsing to the bottom of the diffusion flask by means of a pipette of about 200 c.mm. capacity. The total volume should not exceed about 1.5 ml., forming a thin layer over the bottom of the flask.

On the cup *D* is placed sufficient strong caustic to render the digest solution basic, and the cup is laid aside. On the cup *C* is placed an accurately measured portion of the dilute sulphuric acid in sufficient quantity to provide approximately a 100% excess acid. The flask is warmed by immersing it in a bath of water at about 90 to 100° C., and the cup *D* is lowered into it with a glass hook in such a way that the caustic does not mix with the digest. Immediately, the stopper *B*, carrying the cup *C*, is placed and sealed with stopcock lubricant. The cell is then dipped into a beaker of cold water far enough to just cover the liquid layer in the bottom, the cooling causing a slight vacuum which keeps the cell well sealed. The contents of cup *D* are then spilled by tipping, without disturbing the acid in *C*. While cooling the bottom of the cell in the cold water

..

C.M.A.



the caustic is mixed by swirling, and the cell placed in an oven at about 50° C. On standing for about 2 hours, the ammonia completely diffuses into the dilute acid. The stopper is withdrawn and the cup *C* placed on the titration table. The excess acid is carefully back titrated with the capillary burette to a canary yellow end-point (methyl red indicator).

The titration value is subtracted from the corresponding value run identically when no nitrogen is present in the sample. This difference corresponds to the amount of ammonia formed in the digestion.

The chief source of difficulty in the determination comes from a small amount of acid which creeps up the side and is never adequately neutralised, but this is prevented by the use of the caustic swab. Another difficulty arises in the heating of the flask previous to closing it with the stopper. It is found that heating in an unstirred air bath causes some condensation of water droplets on the upper walls of the flask due to disproportionate heating of the bottom of the flask. Some ammonia becomes subsequently absorbed in these droplets and thus escapes determination (see comment below). The short immersion in the hot-water bath warms the walls of the flask rather than its liquid contents, and prevents the formation of the undesirable condensate. Spattering is liable to occur during the neutralisation of the digest, and is prevented by cooling the contents of the flask at this stage. Incorrect results will result from spattering at any time during the determination. With the proper observation of the above precautions, the method has been found entirely satisfactory.

(With regard to the possible error, commented on by Kirk and Bentley, occurring from the formation of droplets of condensed water on the upper walls, such droplets will cause appreciable error only when they are slightly acidic, as otherwise any ammonia entering them will exert a tension and will very rapidly diffuse from such small volumes and large surfaces into the acid where zero tension exists. In the ordinary use of the Unit no appreciable error will result from their occurrence.)

**Results of the method.** These may be briefly summarised as follows :

TABLE XI

	<i>Amount of ammonia γ N.</i>	<i>Error as st. dev. (%).</i>	<i>No. of observa- tions.</i>	<i>Maximum error (%).</i>
Ammonium sulphate solutions	4.7	0.6	10	0.8
Ammonium sulphate digests	4.6	1.0	6	1.5
Pure compounds—full procedure	3–10	1.2	12	2.0

## CHAPTER XIV

### UREA

Determination of urea in 0.2 ml. blood. (Revised method.)

#### Procedure.

*Collection of blood (human subject).* This is conveniently taken from the finger, using for the small incision a Franke spring lancet (see Fig. 26), which is very efficient and practically painless. The blood may be squeezed out into a small dry oxalated tube. This tube, of about 1 ml. capacity, is prepared by pouring into it some saturated potassium oxalate and then emptying out. The residual fluid on the walls forms a fine layer of the oxalate on drying either in the oven or quickly over a Bunsen flame. About 0.5 ml. blood may be collected as a rule, allowing duplicate 0.2 volumes for analyses. If the blood is not immediately analysed or is being sent by post, the tube is closed by a small rubber cork.

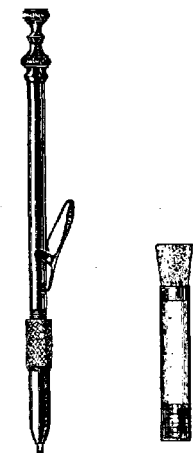


FIG. 26. Franke's lancet, and collecting tube for blood urea determination.

*Preparation of Units.* The acid and alkali solutions of Range I (Table XII) are used with vaseline or paraffin fixative (see also page 81). The central chambers of the Units, cleaned in the manner already described (page 10), are filled with 1 ml. volumes of the  $N/150$  hydrochloric acid containing the indicator, the lids being smeared with the vaseline or paraffin fixative, as given on page 77.

*Urease action and ammonia determination.* Into the outer chamber is introduced 0.2 ml. of the blood from an Ostwald pipette. The pipette may then be washed out once or twice

(but not more) with distilled water and 0.5 ml. of the urease-phosphate mixture run on to the blood. (Alternatively the urease-phosphate solution itself may be used as the wash water).

TABLE XII. *Suitable solution strengths in determining urea*

Range	Acid with indicator in central ch.	Ba(OH) <sub>2</sub> solution in burette.	Each large div. on burette (0.01 ml.) as γ urea.	Max. urea γ.	Applications.
I	1 ml. N/150 HCl.	33.3 ml. N/10 to 250 ml.	4.0	200	General clinical use.
II	1 ml. N/200 HCl.	33.3 ml. N/10 to 500 ml.	2.0	150	Research on blood urea, etc.
III	1 ml. N/1000 HCl.	16.7 ml. N/10 to a litre	0.5	30	Tissue urea, etc.

After placing the lid—smeared with fixative—in position the contents of the outer chamber are mixed by rotation in the usual way.

The Unit is now left aside on the bench for 15 minutes, after which time 1 ml. (approximately) of saturated potassium carbonate is added in the usual manner. It is added without blowing and with the lid slightly displaced. After replacing the lid the Units are left aside on the bench for 1½ to 2 hours. The lid is then detached and the acid in the central chamber titrated until the red colour has just gone or a faint green appears. A blank with the reagents is run at the same time, and an occasional control determination with 50 mg. urea/100 ml. is also advisable.

*Calculation.* With the standard solution used this is very simple—the large divisions on the burette corresponding to the absorbed ammonia are multiplied by 2 and the result corresponds to mg. urea/100 ml. blood.\* A correction for the pipette if this does not deliver 0.2 ml. exactly is unnecessary when the ratio of the blood and urine concentrations is being taken as in renal function tests.

\* With Range II solutions the large divisions on the horizontal burette give directly mg. urea/100 ml. blood.

**Solutions, etc., required for the blood urea determination.**

1. *N/150 hydrochloric acid with indicator.* To make a litre of this solution 10 ml. of the stock Tashiro reagent are run into a litre flask, then 200 ml. of absolute alcohol followed by distilled water until the fluid is about 900 ml. roughly. The mixture is now neutralised to the end-point colour. This will generally require the addition of some dilute alkali or barium hydroxide solution dropped in. After this 66.7 ml. of *N/10* hydrochloric acid are added and the mixture filled to the mark and mixed well.

The *N/200* hydrochloric acid solution with indicator recommended in Table XII for research purposes on blood urea is made up in a similar manner, adding 50.0 ml. of the *N/10* hydrochloric acid.

2. *N/75 barium hydroxide.* 33.3 ml. *N/10* barium hydroxide to 250 ml. (Table XII.)

The *N/150* recommended for research is made by diluting to 500 ml. instead of 250 ml.

3. *Saturated potassium carbonate.* 112 g. of the anhydrous salt may be weighed out for every 100 ml. water added.
4. *Fixative.* Vaseline of best quality may be used, but if this is found to contain ammonia in appreciable amounts a pure paraffin fixative should be used, of a kind containing 50 g. solid paraffin (M.P. about 49°) in 80 ml. of liquid paraffin. (*Vide* p. 81.)

5. *Urease solution (glycerine extract of jack bean).* A preparation found entirely satisfactory in this laboratory is a glycerine extract of jack bean prepared in the manner described by Schmidt (85). 22 g. of finely powdered permutite are washed with 2% acetic acid, which is then decanted off, the permutite being subsequently washed twice with distilled water. The permutite is mixed with 45 g. of finely powdered jack bean meal and 75 ml. of distilled water. These are shaken for about 15 mins. and then 225 ml. glycerol added and mixed. The whole is filtered and the filtrate collected.

It filters slowly, but if a large folded filter paper is used, the whole may be poured on at once and allowed to filter overnight, when a considerable fraction of clear filtrate will have come through, the remainder being allowed to collect over some days. When the filtrate is collected it is stored in the refrigerator in a stoppered bottle. It will keep for several months at least. An advantage of this extract is that, besides being very active, it can be measured out volumetrically without the trouble of weighing very small quantities as with the solid preparation; at the same time any active preparation of urease will be found

suitable. Besides the jack bean, the soya bean may also be used for the extract but is not so potent, and apparently also the seeds of the water melon, as reported by Damodaran and Sivarakrishnan.

6. *Phosphate solution.* 3 g. of anhydrous sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 2 g. of anhydrous potassium acid phosphate ( $\text{KH}_2\text{PO}_4$ ) are weighed out, dissolved in water and made up to 100 ml. (Both salts are stocked as Analar reagents by the British Drug Houses.)
7. *Mixed urease and phosphate solutions for immediate use.* 1 ml. of the phosphate and 1.0 ml. of the urease solution are diluted to 10 ml. and 0.5 ml. of the mixed fluid taken for each determination. Smaller or larger amounts of this mixture are made up according to requirements.

**Accuracy of the method with 0.2 ml. blood.** The standard deviation of the single determination of urea in a standard solution of 50 mg./100 ml. was found to be 0.23 mg./100 ml., using the hydrochloric-barium-hydroxide procedure. This is a rather marked advance on the method as previously described, the standard deviation being then given as 0.6 mg./100 ml. The difference is apparently due to the introduction of the horizontal burette combined with the use of barium hydroxide for titration, the incorporation of the indicator in the acid, etc. This error practically corresponds to that resulting from the pipette (which was used to deliver without washing out) and titration errors alone, which under the conditions would be approximately 0.43%. It may be said, therefore, that the error of this determination depends only on the glass error, and this can be almost indefinitely lessened in accordance with the principles of Part III. *The method, in short, can be made to give almost any accuracy we wish.*

**Comparison of accuracy with that in the aeration method, using 2-3 ml. blood.** The accuracy of the aeration method has been expressed by Addis and Watanabe (86), and later by Kay and Sheehan (87), as mean differences between duplicate analyses. For the urea determination in blood as described above, with the first 150 duplicates in the protocols the mean value of the difference was found to be 0.3 mg./100 ml. Using 3 ml. blood as in the Van Slyke and Cullen (88) method, Addis and Watanabe in 1916 carried out a large number of estimations

and found with 90 duplicates an average difference of 1.5 mg./100 ml. Later they succeeded in reducing this to 0.5 mg./100 ml. Kay and Sheehan in a recent paper have introduced into this aeration method certain improvements in technique designed for greater accuracy. Using 2 ml. blood, they found a mean difference for duplicate analyses of 0.4 mg./100 ml. Some of their experimental blood ureas were very high, but they state that this apparently did not affect the duplicate differences.

This statement of Kay and Sheehan may be taken as it stands for the normal blood urea concentration, for which the micro-diffusion method is more accurate with only one-tenth the amount of blood used, as in ordinary routine working we have found a mean duplicate difference of only 0.3 mg./100 ml.

With regard to the very high blood ureas examined by them they have comparatively few observations with a recovery of only 96%, and for this reason, and also what we know of the errors of titration, etc., an independence of duplicate differences to height of blood urea may be regarded as very improbable.

**The efficiency of urea recovery in the micro-diffusion method.** For the aeration method Kay and Sheehan found only 96% recovery, this being subsequently increased to 98-99% by Kay and Reid (89) using much greater aeration volume. The recovery of urea added to whole blood has been shown to be quantitative in the micro-diffusion method (2). Gibbs and Kirk (90) have confirmed these results, recovering the quantitative amount with as little as 0.00279 mg. urea *N*. Rappaport and Gutman (37) also confirm. Lee and Widdowson (91) have found a mean 99.6% recovery with urea added within the clinical range, and very recently Abelin finds the recovery quantitative using the boric-sulphuric technique. Gibbs and Kirk, moreover, have demonstrated the agreement between urea whole blood analyses and corresponding protein-free filtrates, which they emphasise as a special advantage of the method.

**Clinical use and applicability for renal function tests.** The revised method makes it particularly suitable for renal function tests and also for general clinical use, the use of barium hydroxide removing the need of carbon dioxide free

alkali, and the incorporation of the indicator with the acid being a greater convenience. The accuracy of the method depends purely on one's ability to deliver 0.2 ml. blood and to titrate 1 ml. acid, and there is scarcely any limit to the accuracy with which this can be done when the principles in Part III are understood.

For general clinical purposes it has been pointed out by Lee and Widdowson (97) that the range is rather restricted, being 100 mg. urea/100 ml. Occasionally hospital cases exceed this value and a second determination may be necessary. A second determination may be avoided in all but the very rarest instances if 0.1 ml. and 0.2 ml. blood are used as a routine instead of a 0.2 ml. duplicate.

*It is to be noted that increased ammonia has never been observed from the action of the arginine-arginase system in the method as carried out here.*

The use of the urinary and blood urea concentrations in determining renal function is given in the Appendix.

#### The pH and salt effects on the urease action.

*The pH effect.* Van Slyke and Zacharias (92) very fully investigated the general pH and salt effects on the urease action. Kay and Reid (89) have recently examined the pH effect on total recoveries. These worked, however, on comparatively large amounts of urea compared with the Unit range, and it was thought desirable to investigate again the question in relation to the present method of determining urea.

In the investigation the outer chamber in a series of Units contained 0.2 ml. of a 75 mg. urea/100 ml. solution, 0.5 of a 1 in 10 glycerine extract of urease as previously described under the blood urea determination, the inner chamber containing 1.2 ml. of *N*/200 acid (Range II). The diluted glycerine extract also contained phosphate in *M*/20 concentration of varying pH. The action was allowed to take place at room temperature (18° C.) and interrupted with 1 ml. saturated carbonate after a certain time period.

As may be seen from Fig. 27, *the optimum pH is somewhat to the alkaline side of neutrality*, but there is very little difference between the action at a pH of 7.0 and 8.0. There is a rapid



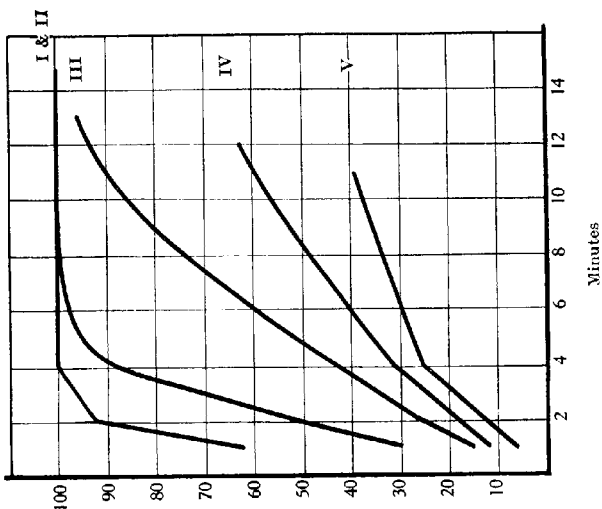


FIG. 28. Time course of urea hydrolysis with different phosphate concentrations at constant pH of 6.8, the mixture being otherwise the same as in Fig. 27

- I. No phosphate added.
- II.  $M/20$  phosphate in the urease solution.
- III.  $M/10$  phosphate in urease solution.
- IV.  $M/4$  phosphate in urease solution.
- V.  $M/1$  phosphate in urease solution.

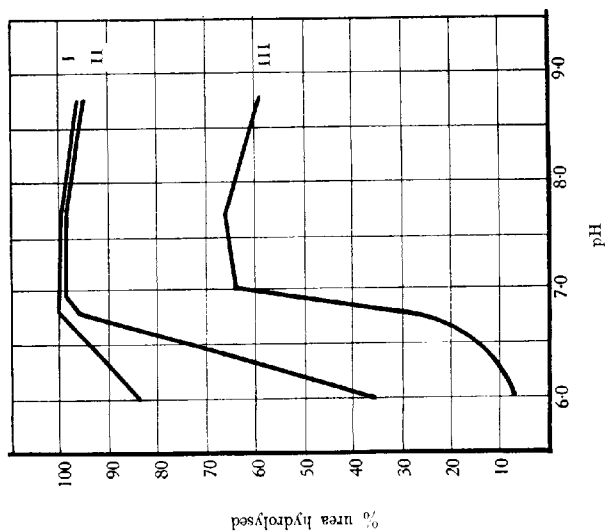


FIG. 27. pH effect on the urea hydrolysis. 0.2 ml. of 75 mg. urea/100 ml. solution plus 0.5 ml. urease solution containing  $M/20$  phosphate at varying pH.

- I. 10 minutes action.
- II. 5 minutes action.
- III. 1 minute action.

fall in activity to the acid side of 7.0 pH, but only a slow fall as the pH rises beyond 8.0.

*The salt effect of the phosphate.* In this investigation the same procedure was adopted, except that the strength of the phosphate was varied at a constant pH of 6.8.

It will be seen (Fig. 28) that *the salt action of the phosphate is very marked, and to economise the time of action it is inadvisable to have a stronger phosphate solution than M/20 in the 0.5 ml. urease-phosphate mixture added.* The question of entirely omitting the phosphate was in fact considered since the rate appears to go as rapidly without it for the small amounts of urea hydrolysed. It was decided to include the buffer in its present composition (as described under the blood urea method) since it ensures against any wide change in the pH value.

**Distribution of the normal blood urea in man.** Where blood ureas are being determined it is often of interest to compare such with the normal distribution. Fig. 29 (C) shows the distribution of the normal blood urea figures for the human subject. The distribution is for 169 determinations in the literature from the figures of Addis and Watanabe (86), Walker and Rowe (93), Austin *et al.* (94), McLean (95), Conway, O'Connor and Donovan (96) and some unpublished data from the author's laboratory. A maximum of five observations was allowed for any single subject, the first five listed being taken. It is to be understood that the subjects were not only normal in renal functioning, but normal in general health, hospital cases being excluded. The mean value of the 169 observations is 31.0 mg./100 ml. with a standard deviation of 7.5 mg./100 ml. It will be seen from the figure that the distribution does not correspond to a 'normal' distribution, but tends to show groupings of results in about three regions. This effect, if confirmed by more extended study, may be largely due to the rise following meals or protein intake, but may also be associated with other metabolic causes. The distributions (A, B) for single subjects investigated in the author's laboratory, for whom there is a large number of determinations, are interesting to compare with the general histogram. It has happened

accidentally that the two subjects investigated had mean blood urea values in the upper and lower regions of the general histogram, the mean for one being 40.2/100 ml. with a

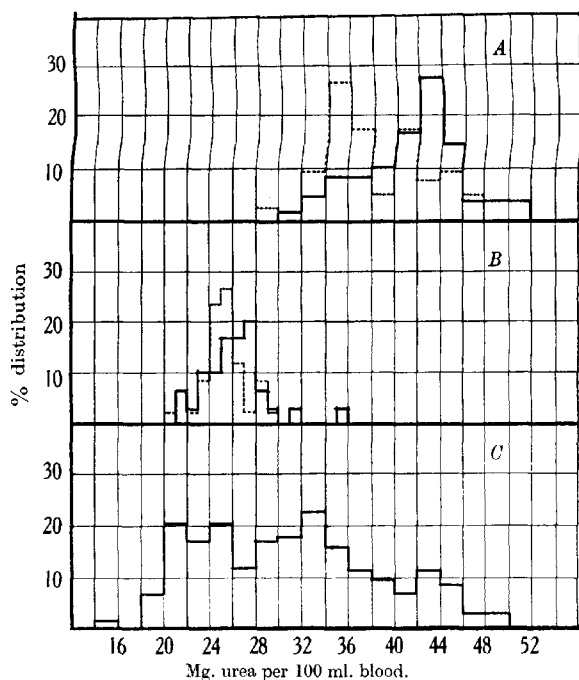


FIG. 29. Distribution of normal human blood urea.

*A*—122 obs. on subject D. O'D. Dotted line—obs. before breakfast.

*B*—64 obs. on subject E. C. 34 before and 30 after light lunch. (Dotted line—before meal.)

*C*—169 obs. from general literature.

standard deviation of 4.7 mg./100 ml. (122 observations) and for the other 26.8 mg./100 ml. with a standard deviation of 3.4 mg./100 ml. (64 observations).

Fasting values for D. K. O'D. (before breakfast and 3–5 hours after meals) gave an average of 38.4 with a standard

deviation of 4.3 and the fasting values of E. J. C. gave 25.9 with a standard deviation of 2.5 mg./100 ml.

The histograms (A, B) show very clearly the influence of meals, which for D. K. O'D. shifts the mode or peak 8 mg./100 ml. and for a light meal with E. J. C. raises it 2 mg./100 ml.

**Determination of the urinary urea.** This determination is usually carried out in conjunction with that of the blood urea, and is done most conveniently after suitable dilution, so that the same solutions, pipettes, etc., may be used.

Seeing that the concentration in the urine is related to the rate at which urine is excreted, this latter must be considered in accurate working, the dilution being so made that a considerable fraction of the absorbing acid is always neutralised by the formed ammonia. Suitable dilutions may be made from the data in Table XXXVI, giving average concentration ratios of urine/blood in the varying urine volumes.

After diluting, the urine is analysed in the same manner as the blood, 0.2 ml. being introduced into the outer chamber, 0.5 ml. of urease phosphate solution added, etc.

One difference between the blood and urine determinations consists in the fact that even the diluted urine contains a small concentration of preformed ammonia which will amount to about 7% of the ammonia from the urea at 25 ml. per hour, 5% at 50 ml. and about 3% at or greater than 150 ml. In contrast with the urine the blood contains no ammonia on shedding (5), and with human blood no appreciable ammonia compared with the urea content up to 2 hours after shedding. (After 24 hours, sterile human blood will contain preformed ammonia corresponding to about 1 mg. urea/100 ml. blood and formed chiefly by the breakdown of adenylyl pyrophosphoric acid.) Consequently it is necessary in urinary urea determinations to set up another Unit with 0.2 ml. of the diluted urine in the outer chamber, 1 ml. of saturated potassium carbonate being added as usual and the ammonia found subtracted from the total.

#### Calculation.

EXAMPLE. Urine rate - - - - - 53 ml. per hour.

(From Table XXXVI the mean urinary concentration at this rate is

56.8 times that of the blood. A dilution of 50 times may therefore be made. (*Vide* Appendix and the Normality Ratio.)

Solutions of Range II. Table XII, p. 117.

Analytical Unit	-	-	-	41.35	large	divs.
Blank Unit (urine)	-	-	-	73.50	„	„
Blank Unit (urease)	-	-	-	75.65	„	„
1 fill of acid	-	-	-	76.05	„	„
Preformed ammonia in dil. urine	-	-	-	$(76.05 - 73.50) = 2.55$ large divs.		
Preformed plus urea ammonia	-	-	-	$(75.65 - 41.35) = 34.30$ „ „		
Urea ammonia	-	-	-	$(34.30 - 2.55) = 31.75$ „ „		

Urea in original urine is therefore

$$31.75 \times 50 \text{ mg./100 ml. urine}$$

$$\text{or } 1.587 \text{ g./100 ml.}$$

Pipette and alkali corrections (75/76.05 for the alkali) are made as usual, but if the ratio of blood and urine concentrations are being taken it is unnecessary.

**Distribution of urinary urea concentrations in the normal human subject pursuing an ordinary living routine.** Unlike the urea concentration in the blood, that in the urine is very dependent on the rate at which the urine is being excreted. The distribution of the urinary urea for a number of normal subjects is given in Fig. 30 (C), and similar distributions for the two normal subjects considered under the blood urea. What is at first peculiar about the three distributions is their rather marked similarity, seeing that the blood distribution is very dissimilar, particularly for the two single subjects, and also that the urine rate has a very marked effect on urinary concentration. The main bulk of the distribution lies in each case between 1.8 and 2.8 g./100 ml., with the chief mode at 2.1, 2.3 and 2.3 g./100 ml. respectively. In each case there is an appearance of a higher mode at about 2.6 g./100 ml., this latter being due very likely to the influence of meals as shown by dotted line in distribution B for one subject (E. J. C.). The appearance of a third mode at a lower concentration level is obviously due to diuresis, and from that mode downwards we have scattered observations due to high diuresis.

**Distribution of the normal urine volume.** It is of interest in connection with the other distributions and in relation

to the question of urine dilution for urea analysis, to consider also the distribution of normal urine rates, under

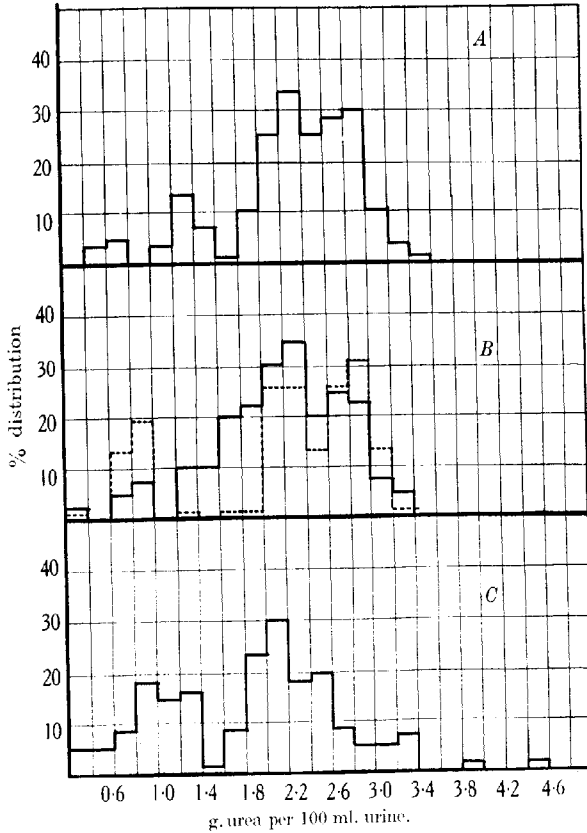


FIG. 30. Distribution of urine urea concentrations in the normal human subject. Water-drinking to produce diuresis excluded.

A—120 obs. on subject D. O'D.

B—81 obs. on subject E. C. Dotted line gives obs. after light lunch (37).

C—111 obs. from general literature.

apparently normal conditions. As shown in figure the distribution is similar to that of the urine urea, but is inversely

related, high rates being associated with low concentrations and vice versa. From the distributions in Fig. 31 (corre-

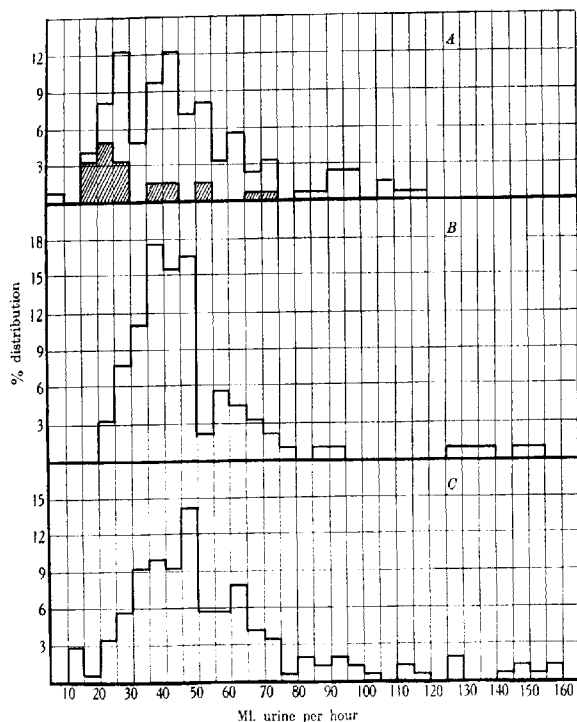


FIG. 31. Distribution of urine volume in normal human subject. Water-drinking to produce diuresis excluded.

A--obs. on subject D. O'D. as in Fig. 30. Shaded section--obs. before breakfast.

B--obs. on subject E, C. as in Fig. 30.

C--obs. from general literature as in Fig. 30.

sponding to previous Figs. 29 and 30, A and B being for the same two subjects and C the general distribution) data after water drinking with the intention of producing diuresis are excluded. The main mode of the three histograms for the general group and two subjects lies around 40 to 50 ml. per

hour, corresponding approximately to 1 litre per day. It is around this point therefore that we can expect to have most observations in the normal subject. There is a tendency to a subsidiary mode at about 62 ml. per hour, corresponding to about 1,600 ml. per day.



## CHAPTER XV

### DETERMINATION OF UREA IN TISSUES

*Principle.* The tissue is ground with water and a little pure quartz sand, largely cleared by centrifuging and the urea hydrolysed in an aliquot part by the action of urease as with the blood and urine determinations, the remaining procedure being also similar.

*Procedure.* The tissue section for analysis (100 to 200 mg. are usually suitable, though the amount may be varied greatly) is weighed in a covered watch glass and then transferred to a small mortar, 3 ml. of distilled water added from a standard pipette and a little pure quartz sand (Merck's). The tissue is ground to a fine suspension, the mixture poured into a small centrifuge tube and spun for a minute or two.

The following Units are then set up, using the solutions of Range III (Table XII, p. 117) :

Analytical Unit (urease plus extract) -				outer chamber—0.5 ml. extr.
				plus 0.5 ml. urease phosphate mixt. (as for blood urea)
Blank Unit (extract) -	.	.	.	outer ch.—0.5 ml. extr.
Blank Unit (urease) -	.	.	.	outer ch.—0.5 ml. urease-phosphate.

The inner chambers of the Units are filled with 1 ml. of the absorbing acid—made up as described for ammonia determinations.

After 15 minutes on the bench 1 ml. of saturated potassium carbonate is added to each Unit and the inner chamber titrated after two hours' absorption. (If it is thought very desirable to reduce the time required for this, twice the amount of tissue may be used in the grinding, and 0.2 ml. extract with 0.2 ml. urease-phosphate used instead of the above proportions, then

1 ml. carbonate. Only 1 hour need then be given for the absorption at room temperature.)

It is advisable here as always to work with duplicates for each procedure. Such procedure as outlined above will no doubt require suitable modification in accordance with the experimental conditions, which may involve the injection of urea.

*Calculation.*

*Urea in 1 ml. extract.* (0.5 ml. used.) When the titrating fluid in the burette is made up as 16.7 ml.  $N/10$  to the litre, each large division (0.01 ml.) is equivalent to 0.5  $\gamma$  urea. Since 0.5 ml. of the extract is used, it follows that the number of large divisions on the burette corresponding to the urea ammonia gives the  $\gamma$  urea per ml. extract.

This is illustrated by the following example :

Analytical Unit (urease plus extract)	-	43.7	large divs.
Blank Unit (extract)	-	59.4	„ „
Blank Unit (urease)	-	59.5	„ „
1 fill acid	-	60.0	„ „
Urea ammonia in extract,	$16.3 - 0.6 - 0.5 =$	15.2	„ „

or 15.2  $\gamma$  urea/ml. extract (0.0152 mg./ml.).

*Urea in 100 g. tissue.* (0.150 g. used, for example.) It will be assumed that the tissue contains 80% water, though small deviations from this will not appreciably affect the result. The total amount of fluid in the mortar is therefore 3.0 plus 0.8 $w$ , where  $w$  is the weight of the tissue in g.

Each g. tissue corresponds to  $\frac{3.0 + 0.8w}{w}$  ml. extract and 100 g. tissue to  $\frac{3.0 + 0.8w}{w} \times 100$  ml. extract.

Hence if  $U$  is mg. urea in 1 ml. extract, the amount of urea in 100 g. tissue is given by

$$U \times (3.0/w + 0.8) \times 100.$$

When  $U$  is 0.0152 as above and  $w$  is 0.150 g. we get the result as—31.6 mg./100 g. tissue.

**Note on kidney tissue.** Where the kidney tissue is being analysed it is necessary to boil the mixture and cool prior to taking a sample for analysis (in order to kill the ferment which

forms extra ammonia). Usually it will be found most convenient to pour the mixture—washing out residue—into a small flask and heat to boiling, cool and make up to a special volume such as 10, 25, etc., and then take 0.5 or 1 ml.

The original weight of the tissue may be varied to suit Range II analysis. Alternatively the mixture may be brought momentarily to boiling in a small pyrex tube, and subsequently immersed in boiling water for a few minutes, the volume if necessary being made up to the original—the level having been marked with a file. The amount of urea in the tissue water in general corresponds to that in the blood plasma, notable exceptions being brain and cord tissue, urea being present in the former to only 52% of the plasma concentration and in the latter to only 5–10% (97).

## CHAPTER XVI

### ADENYLPYROPHOSPHORIC ACID, ADENYLIC ACID, ADENOSINE, ETC.

SUBSEQUENT to the recognition of adenylic acid in blood by Bass (98), it was discovered by Embden in muscle (99). Lohmann showed, however, that it did not exist as the free adenylic acid in muscle, but as a compound with pyrophosphoric acid (100), termed adenylypyrophosphoric acid or briefly A.T.P. In blood the adenylic acid also exists similarly bound. Adenylypyrophosphoric acid is intimately connected with the phosphorylation of hexose in its conversion to lactic acid, and as is well known, a similar but not identical substance exists in yeast (*vide* Schmidt, 101).

Adenylypyrophosphoric acid breaks down rapidly on the death of the animal, and if it is to be determined in muscle this must be excised immediately after death, being ground in ice-cold trichloroacetic acid. It is also unstable in alkaline and in strongly acid media. In muscle it exists to the extent of about 4-6 mg. of amino N per 100 g., and in human blood, as determined by the author (52) to the extent of 1.0 mg. amino N/100 ml., being in similar concentration in rabbit blood, and agreeing with the nucleotide data of Buell (102).

**Principles of determination.** In the ensuing description the method described by Parnas (103, 104) is followed, it being adapted for Unit determinations, using the solutions of Range II (p. 76).

The adenylypyrophosphoric acid in the muscle passes to a constant proportion of 90% into the trichloroacetic filtrate when the described proportions are used, the remaining 10% being present in the precipitate. On bringing the filtrate to a pH of 8.5 and adding excess of barium acetate the A.T.P. is precipitated as the insoluble barium salt, any adenylic acid, if present,

being left in the fluid. In fact, however, adenylic acid as such will not be found in the fresh muscle.

The insoluble barium salt is subsequently dissolved in acid, the barium removed by sodium sulphate and centrifuging, and after neutralising, etc., is acted upon by some fresh muscle extract and the ammonia formed is determined.

In this way two special qualities of the A.T.P. are both availed of to ensure that this substance only is being determined. These are the insolubility of its barium salt at a particular *pH* and its specific deamination by the extract.

*Procedure.* The freshly excised muscle is quickly weighed and introduced into an ice-cold mortar with pestle. From a graduated 5 ml. pipette 10% ice-cold trichloroacetic acid is added 1 ml. for every g. muscle and the muscle ground quickly with the aid of a little pure quartz sand (Merck's) to a fine suspension.

It may be noted here that all solutions used down to the solution of the barium salt in acid should be ice-cold like the trichloroacetic acid. Water is now added in the same volume as the acid, and the mixture is left for at least fifteen minutes in the refrigerator with frequent stirring. It is then poured into a centrifuge tube and quickly spun. 4 ml. of the clear fluid (corresponding to 1.43 g. tissue) are taken in a centrifuge tube and a drop of phenolphthalein solution added and the mixture carefully brought to a faint pink (about *pH* of 8.5) by the addition of 0.1*N* NaOH, the tube being immersed in ice. 1 ml. of 25% barium acetate, which has been carefully neutralised, is now added and the mixture left aside in the refrigerator for 30 minutes, then centrifuged and washed twice with 1% neutral barium acetate.

The precipitate is then dissolved in 2.5 ml. of 0.1*N* HCl, the barium removed by adding 1 ml. of 15%  $\text{Na}_2\text{SO}_4$  and centrifuged. The clear fluid is poured into a 10 ml. measuring cylinder, the residue being mixed with 1 ml. water and a small drop of the sodium sulphate solution, centrifuged and the fluid poured into the cylinder.

The fluid in the cylinder, which is neutralised carefully with 0.1*N* NaOH (using bromthymol blue), of which upwards of 2

ml. will be required, 0.1 ml. of  $M/3$  phosphate ( $pH$  7.0) and the mixture brought to the 10 ml. mark with water.

Three glass tubes of 5 ml. capacity provided with stoppers are now prepared and into these are introduced the following :

- Tube 1 - 2 ml. of fluid (from cylinder) plus 0.2 ml. muscle extract.
- Tube 2 - 2 ml. of fluid plus 0.2 ml. water.
- Tube 3 - 2 ml. of water plus 0.2 ml. extract.

The three tubes are incubated at  $38^\circ$  for 1 hour. Subsequently 1.0 ml. volumes are pipetted into the outer chamber of prepared Units containing 1 ml. acid of Range II, made up as on page 79, and 1 ml. of saturated potassium carbonate added, the Units being titrated after  $1\frac{1}{2}$  hours at room temperature (or after  $1-1\frac{1}{2}$  hours at  $38^\circ$ ). The amount of adenylypyrophosphoric acid amino-nitrogen corresponding to 130 mg. of the original muscle is finally analysed in this manner.

*Calculation.* If the ammonia—as large divisions on the burette—in samples from the three tubes be written I, II and III, then the A.T.P. ammonia corresponds to

$$(I - II - III) \times 0.3\gamma \text{ ammonia } N.$$

This corresponds to the A.T.P. amino  $N$  in 130 mg. muscle, but under the conditions—retention of 10% A.T.P. in the trichloroacetic precipitate—the result must be multiplied by 1.11. Milligrammes A.T.P. amino  $N$  in 100 g. muscle is then given by

$$(I - II - III) \times 0.256.$$

*Solutions required.*

- 10% trichloroacetic acid.
- 25% barium acetate and 1% barium acetate (both brought to 8.5  $pH$  or faint pink with phenolphthalein).
- 0.1  $N$  HCl.
- 0.1  $N$  NaOH.
- 15%  $Na_2SO_4$ .
- Phosphate buffer,  $M/3$   $pH$  7.0 (3 volumes of  $M/3$  alkaline to 2 volumes of  $M/3$  acid phosphate).

Muscle extract 1 g. in 10 ml. of  $M/3$  phosphate ( $pH$  7.0).

The muscle is ground to a fine suspension with quartz, then the mixture is largely cleared by centrifuging.

Acid and alkaline solutions of Range II (pages 76 and 79).

The method can obviously be scaled down to determinations in lower amounts and single frog's gastrocnemii could be used.

**Adenylpyrophosphate in blood.**

*Principle and Procedure.* This determination is carried out in practically the same way as for muscle. The blood may be received into a graduated cylinder containing about one-tenth of the expected volume of 1.3% potassium oxalate. After collecting and mixing the exact volume is read. The blood is then laked in the same volume of water. To 10 ml. of the laked blood is added 5 ml. 10% ice-cold trichloroacetic acid and the mixture centrifuged. 10 ml. of clear fluid are pipetted into a 25 ml. centrifuge and a drop of phenolphthalein added, and the mixture brought to a faint pink, proceeding firstly with normal NaOH then with 0.1N. 4 ml. of 25% neutral barium acetate are added, and from this onwards the method is identical with that of muscle, not only in general procedure but in volumes and quantities used.

*Calculation.* Similar to the previous method. The A.T.P. amino-nitrogen analysed corresponds here to 0.5 ml. approximately of original oxalated blood. If 1.3% potassium oxalate has been used to prevent clotting, this must also be allowed for (as also the fact that 1 vol. blood contains 0.85 vol. water).

*Solutions required.* Same as for muscle, with addition of 1.3% potassium oxalate (ammonia-free).

**Determination of adenylic acid in the presence of A.T.P. in muscle and blood.** If free adenylic acid is present it is left in solution after the precipitation out of the barium salt of the A.T.P. Suitable methods similar to that of the A.T.P. determination may therefore be devised, the barium being removed by sulphuric acid with subsequent neutralisation, etc.

**A short method for determining the total A.T.P. and adenylic acid in blood.**

Since there is no appreciable amount of free adenylic acid in freshly shed blood, this may be also taken as a short method for determining the A.T.P. of blood.

*Principle.* The blood is acted on directly by muscle extract, the preformed ammonia in the blood itself and extract under similar conditions being also determined.

*Procedure.* The following solutions are introduced into small stoppered Erlenmeyer flasks.

*Flask I.* 5 ml. laked blood plus 1 ml. buffer (maleic acid) plus 4 ml. muscle extract (1/10 with 0.6 NaCl).

*Flask II.* 5 ml. laked blood plus 1 ml. buffer (maleic acid) plus 4 ml. water.

*Flask III.* 5 ml. water plus 1 ml. buffer (maleic acid) plus 4 ml. muscle extract.

Flask II is analysed for ammonia at once after setting up, and flasks I and III after 1 hour at room temperature.

In the analysis 1 ml. fluid is pipetted into the outer chamber of a prepared Unit, 1 ml. saturated carbonate added and the Unit left for  $1\frac{1}{2}$  hours at room temperature. (Solutions of Range II used.) (0.5 ml. volumes may also be used with solutions of Range III (p. 76), 1 ml. being placed in the central chamber instead of 0.7 ml. as usual with this range.)

The total adeny amino *N* is given as mg./100 ml. original oxalated blood by

$$(I - II - III) \times 4 \times 0.0003 \times 100,$$

$$(I - II - III) \times 0.12,$$

where I, II and III represent the ammonia from flasks I, II and III in large divisions on the burette, using alkali of Range II (Table V). It is supposed in this calculation that the blood, as before, has been laked by an equal volume of distilled water. If the 1.3% oxalate solution is used to prevent clotting, allowance must be made for this in the final calculation.

In the above determination about 10 large burette divisions may be expected to correspond to the adeny amino *N* in the blood. Much smaller volumes of blood may be used with similar accuracy.

The results with this method are similar to the Buel and Perkins determinations of total adenine nucleotide.

*Solutions required for short method.*

Maleic acid buffer, 20 c.c. 5.8% maleic acid plus 17.3 ml. N/1 NaOH.

1 in 10 muscle extract with 0.6% NaCl. The muscle is ground with some quartz sand and saline, then cleared by centrifuging.



**Determination of other amino purine derivatives by the present technique.** The possibility of these determinations may at present be only indicated.

**Adenosine.** Whole or laked blood deaminates adenosine with great rapidity and specificity, the process being complete in about 15 minutes at a pH of 7.0 and a 1 in 5 dilution of rabbit blood (room temp.) (51, 52). Provided adenylic acid (adenosine 5-phosphate) or adenylypyrophosphate is present in less than 0.1%, with *N*/10 phosphate, it does not appreciably interfere. No ammonia is formed from guanosine, cytidine, adenine, guanine, cytosine, glycine, aspartic acid, tyrosine, tryptophane, leucine, histidine, cystine, histamine, choline, isoamylamine, butylamine, acetamide, propionamide, butyramide, glucosamine, urea, etc. (52).

**Adenine, guanine, etc.** Ferments specific for such substances have been already investigated by many observers (e.g. Jones and Schmidt, refs. 105, 106), and it is found here that a very rich source of a number of such deaminases is the nucleated corpuscles of the fowl. They contain, for example, a high concentration of adenase, which is otherwise rare; also guanase, and ferments deaminating cytidine, cytosine, etc. (52).

## CHAPTER XVII

### VOLATILE AMINES

THE lower aliphatic amines are volatile substances, resembling ammonia itself in many properties. Trimethylamine is important biologically as associated with bacterial decomposition. As a test for such it has been used in the Atlantic and Pacific Experimental Fisheries Stations. A method for determining trimethylamine—using the standard Unit—has been worked out by Beatty and Gibbons of the Atlantic Station (107) and is described below.

The test was also used by Brockelsby and Riddell of the Pacific Station (Prince Rupert, B.C.) in studies on iced halibut (108).

A very interesting paper by Richter (7) has been recently published in which the metabolic importance of these lower aliphatic amines (methylamine, dimethylamine, ethylamine) becomes evident. It was shown that in the oxidation of adrenaline by the enzyme described by Blaschko, Richter and Schlossmann (109, 110), in liver, intestine, etc., of mammalian tissues methylamine was formed; from epinine and *l-p*-sympatol methylamine was also formed; from *dl*-alkalamine ethylamine and from hordenine dimethylamine.

In the identification and quantitative determination of these amines and ammonia, Richter used the standard Unit.

**Richter's method for the identification of the volatile amines formed in enzymic mixtures** (from the *Biochemical Journal*, 31, 2022):

Ammonia is liberated from the reaction products by treatment with an equal volume of saturated  $K_2CO_3$  in a standard Unit. It is then converted into (a) the picrate, and (b) the 2·4-dinitro-naphthol derivative by introducing about 0·05 mg. of the reagent suspended in a drop of water on a coverslip attached by vaseline to the lid of the standard Unit. For identifying the volatile amines traces of ammonia coming from the enzyme preparation were first removed by keeping the

solution with an equal volume of saturated  $K_2CO_3$  for 45 minutes at 37 degrees in a Unit containing dilute sulphuric acid in central chamber.

In this time 99.5% of the ammonia, but only a much smaller fraction of the amines, passes over into the acid. The residual amine is then converted into the derivatives (a) and (b) as with ammonia, and the derivatives are identified by examining the optical properties of the crystals with the polarizing microscope in plane and polarised light (Klein and Steiner, ref. III). The derivatives may be recrystallised on the cover-slips in order to obtain well-defined crystals.

It is possible to identify small quantities of amines (of the order of  $10\gamma$ ) very rapidly and with a high degree of certainty. Richter lists the following properties for identification, some of which were previously given by Klein and Steiner (III).

TABLE XIII.

	<i>Reagent.</i>	<i>Ammonia.</i>	<i>Methyl-amine.</i>	<i>Dimethyl-amine.</i>	<i>Ethylamine.</i>
<i>Picrates.</i>					
Habit	Prisms	Plates and Prisms	Plates	Plates	Plates and Prisms
Pleochroism	Weak	Distinct	Strong	Weak	Strong
Extinction	Parallel	Parallel	Parallel	42°	Parallel
Interference	Medium	Strong	Strong	Strong	Very strong
Crystal edge angles	$\alpha = 137^\circ$	$\alpha = 117^\circ$	$\alpha = 129^\circ$ $\beta = 141^\circ$ $\gamma = 102^\circ$ $\delta = 78^\circ$	$\alpha = 96^\circ$ $\beta = 132^\circ$	$\alpha = 123^\circ$

## 2 : 4-Dinitronaphthol derivatives

Habit	Prisms	Fine-pointed needles	Blunt-ended prisms, often curved	Short prisms, square-ended	Plates
Pleochroism	Weak	Weak	Yellow-brown	Marked yellow-brown	Weak
Extinction	0-10°	Angular	Parallel	20°	Angular

### Quantitative estimation of volatile amines (Richter, ref. 7).

The absorptions of several volatile amines compared with ammonia are given in Fig. 32 (from the *Biochemical Journal*).

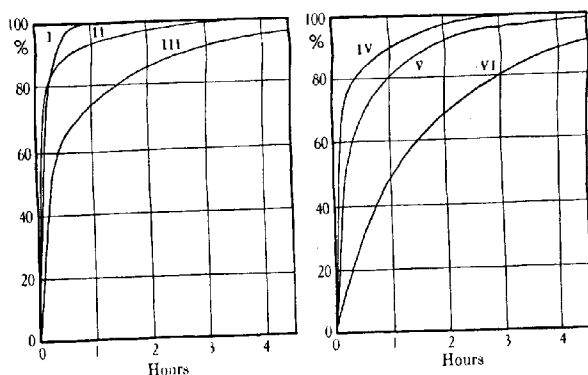


FIG. 32. Rates of absorption of amines. I, ammonia. II, *iso*-butylamine. III, methylamine. IV, *iso*-amylamine. V, ethylamine. VI, benzylamine. At 37° in standard Unit (from Richter's diagrams in the *Biochemical Journal*, 31, p. 2020).

Each Unit contained 1 ml. of 0.015*M* amine hydrochloride and 1 ml. saturated  $K_2CO_3$  in the outer chamber and 2 ml. of *N*/100 sulphuric acid in the central compartments. Richter comments on the fact that isobutylamine and isoamylamine, although much less volatile than methylamine, were absorbed quickly, and considers the probable explanation as due to the lesser solubility of the first two. That this explanation is correct will appear from the considerations in Chapter IV.

Prior to Richter's paper some preliminary observations had been carried out here with regard to the amine absorption rates. These may be summarised provisionally in the following table:

These absorption rates into the central chamber of the Unit were investigated on *N*/250 solutions of the corresponding chlorides, the absorption taking place into 1 ml. of *N*/150 acid (solutions of Range I used). Into the outer chamber was run 1 ml. of the *N*/250 solution of the amine hydrochloride and then 1 ml. of 0.1*N* NaOH or 1 ml. of saturated potassium carbonate.

The latter solution has a very marked effect—as with ammonia—in increasing the tension of the dissolved amine.

The amines listed in Table XIV would be completely absorbed in  $3\frac{1}{2}$  hours at room temperature, when about 10 times the half

TABLE XIV.

Amine hydrochloride (1 ml. N/250 solution in outer ch.).	50% absorption period (mins.).		pH at which the amine is 50% dissociated (112).
	1 ml. 0.1N NaOH added to 1 ml. in outer chamber.	1 ml. sat. pot. carb. added to 1 ml. in outer chamber.	
Ammonia - -	30	10	9.4
Methylamine - -	99	19	10.6
Dimethylamine - -	80	22	10.8
Trimethylamine - -	22	5	9.8
Ethylamine - -	106	16	10.6
Diethylamine - -	86	9	11.0
Triethylamine - -	16	4	10.7

Room temperature 20–23° C.

absorption period with saturated potassium carbonate is allowed. From Richter's figure (Fig. 32), however, the absorption of the last 10% of the methylamine is unexpectedly slow. Trimethylamine and triethylamine go particularly fast, and 1 hour would be sufficient for their complete absorption. The difference between the pH value for half dissociation of the amines gives a means if necessary of an analytical separation. Thus if a dilute borate buffer solution were used sufficient to maintain the pH approximately at 9.4 and the same volumes otherwise used, then after 260 minutes at room temperature (20° C. approximately) the following are the probable order of the amounts absorbed:

Ammonia - - - - -	95%
Trimethylamine - - - - -	90%
Triethylamine - - - - -	73%
Methylamine - - - - -	10%
Ethylamine - - - - -	9%
Dimethylamine - - - - -	8%
Diethylamine - - - - -	5%

For the present the figures of this list must be taken only as very approximate and merely practical guides, since they have

not been actually tested, but derived from calculations on the presumed course of the absorption and the data of the previous table.

**Trimethylamine method of Beatty and Gibbons (107).**

*Principle.* The method was devised for determining trimethylamine in press juice obtained from the muscle of fishes. The reaction between formaldehyde and ammonia is utilised to hold back the ammonia while the trimethylamine diffuses over into acid in the central chamber, the amount being subsequently determined by titration.

*Method.* In obtaining the press juice a press cup of brass is taken which is perforated with holes 1/16 inches (ca. 2.1 mm.) in diameter. The cup is lined with Whatman No. 1 filter paper and filled with the muscle under examination. A circular sheet of filter paper is placed over the sample, a brass plunger inserted and pressure applied. In the inner chamber of the standard Unit is placed a measured excess of standard acid, usually about one-seventieth normal. In the outer chamber is placed 1 ml. press juice and 0.5 ml. formaldehyde solution. Usually commercial formalin, shaken with an excess of magnesium carbonate and filtered or decanted, gives zero, or an extremely low, blank. After the addition of the formaldehyde the lid with fixative is placed in position and the dish rotated to ensure thorough mixing of press juice and formaldehyde. 1 ml. of saturated potassium carbonate is added in the customary manner, the lid being slightly displaced and subsequently replaced.

The dish is rotated to mix the contents and is placed in an incubator at 36° C. for 2 hours, after which the excess standard acid is titrated.

It is obvious that the standard acid may here be made up in the usual way of incorporating the indicator in the standard acid (page 79).

*Suggested modifications.* Instead of using press juice an extract formed by grinding with quartz sand and clearing by centrifuging would apparently work as well as the press juice—using weaker standard acid. Also if 0.5 ml. of this and 0.2 ml. formaldehyde be used the Unit may be left on the bench for

2 hours instead of being incubated. In this way the solutions of Range I or II (Table V, chapter IX) will be found suitable.

*To illustrate the kind of figures obtained*—using the previous method—the following example may be given from the account by Brocklesby and Riddell. The halibut caught 8 hours previously (and presumably stored in the fish-hold, which is approximately at 35° F.) were dressed, iced and boxed, then stored on ice in a cool room. The trimethylamine content in the back muscle just behind the head, which was found to be 0.2 part per 100,000 immediately on arrival, remained very steady up to the ninth day, when it was 0.4 part. On about the twelfth day a rapid change set in, the trimethylamine content being then 1.3 parts, and on the 15th day 12.8 parts per 100,000. With halibut muscle juice (113) stored at 35° the change set in rapidly on the sixth day.

Such changes in the trimethylamine content at 35° F. correspond in time to marked changes observed (114, and unpublished results) in the permeability of the frog's sartorius muscle in Ringer at 2 to 3° C., with respect to sodium, potassium, magnesium and chloride. These permeability changes set in rather suddenly about the 4th to the 6th day.

In the above trimethylamine examination on iced halibut it was found by Brocklesby and Riddell that if the fish were given a five-minutes dip in a 2% formaldehyde solution before icing, after 16 days the trimethylamine content was below 0.8 part per 100,000. No formaldehyde had penetrated the skin, which with the cut surface was also rendered impermeable to the further penetration of bacteria. The experiment illustrates the bacterial origin of the trimethylamine.

## CHAPTER XVIII

### THE HALOGENS (INTRODUCTORY)

THE principle used in the determination of the halogen salts consists in oxidation to the free element in the outer chamber, by oxidants such as acid permanganate or dichromate, and the absorption of these by diffusion in the central chamber in 1 ml. of 20% potassium iodide, where it liberates an equivalent amount of free iodine.

The high percentage of iodide is selected to prevent subsequent diffusion of iodine from the central chamber.

Subsequent to the complete absorption, the free iodine when it exceeds a concentration of  $N/1000$  (35 $\gamma$  chlorine) is titrated with  $N/20$  thiosulphate, a distinct end-point change occurring with less than a small division of the micro burette and sufficiently sharp for obtaining a coefficient of variation of 0.5% with the single determination, using amounts of the order of 0.3 mg. chlorine.

It may be noted that the presence of the 20% iodide introduces a purplish colour into the starch iodide end-point instead of the usual blue, but this introduces no practical difficulty in the titration as described.

Below a level of 35 $\gamma$  chlorine (1 ml. of  $N/1000$ ) it will probably be found more advantageous to use a colorimetric method, and the free iodine colour in the central chamber may be directly determined, or for the lowest levels (less than 7 $\gamma$  Cl), the starch iodide colour determined after starch addition. Though the method has been worked out on the above principle, it is capable of wide variation with respect to possible absorbents and modification of the colorimetry or the titration method, but such need only arise when an attempt is made to exceed the high accuracies already found.

*With the colorimetry at the lowest levels, it is advisable for the worker to plot his own curve of recoveries with respect to the*



*theoretical quantities, as slight and possibly unsuspected changes in procedure may alter a little that already drawn.*

The curves for the very lowest levels were made out with a Unit of a non-alkaline glass before the production of the present pyrex Unit, which may be expected to give, if anything, somewhat better results.

**The titration with thiosulphate.** The thiosulphate used is  $N/20$  and keeps well when a trace of carbonate is added. If controls of 1 ml. of  $N/100$  HCl are occasionally carried out the thiosulphate need not be otherwise standardised. The starch solution used is 0.2% dissolved by boiling, with a minute quantity of mercuric iodide as described in text. Other observers may prefer different solutions, such as cold extract of soluble starch cleared by centrifuging. Any starch solution used, however, should be clear and give a marked end-point change with less than a single small division on the burette.

**Delicacy of the micro-diffusion method of halogen analysis.** The possible delicacy of chloride determinations consists here in how far we can determine photometrically the concentration of free iodine in the iodide using starch solution, or some equally sensitive colour formation with either free chlorine (e.g. with *o*-tolidine) or free iodine.

With 0.5 ml. of 0.1% starch addition to the 1 ml. iodide we get for the free iodine equivalent of 1 $\gamma$  chlorine absorbed an extinction of 0.94 with 50 mm. stratum layer. With 0.7 ml. iodide and 0.3 ml. starch this would become 1.4. The extinction values were determined with Leitz filter No. 3, but higher values can be obtained with filters No. 2 or 1, giving wavebands in the purple, but not so convenient to work with owing to the weakness of this band in the usual light source. The extinction may in short be increased to 2.0.

Since accurate photometric determinations can be carried out with extinctions of 0.25 (5 mm. depth of 'grey solution'), it is possible therefore to determine chloride accurately down to 0.1 $\gamma$  chlorine. This would mean, however, special preparation of Units to decrease blank value further, though this difficulty scarcely arises with bromide; also a further investigation of

the slight initial lag in free iodine formation, as described in the subsequent account of methods, would be desirable.

In the above considerations it was assumed that a 50 mm. stratum thickness was the maximum, using 1 ml. volumes. This limit is set only by the apparatus at present available, but thicker strata with similar volumes would increase the possible delicacy.

## CHAPTER XIX

### CHLORIDE

**Principle and Introductory.** The principle used in the micro-determination of chloride consists in oxidising the chloride to chlorine gas by a suitable acid permanganate mixture in the outer chamber, the chlorine so formed being absorbed by potassium iodide in the central chamber, where it liberates an equivalent amount of iodine.

The formation of the chlorine gas takes place quite readily at room temperature, and its absorption is complete in one hour and a half.

For a range of chloride down to  $35\gamma$  ( $0.035$  mg.) the liberated iodine is best titrated with sodium thiosulphate solution, most suitably by  $0.05N$  solution from the horizontal burette described in chapter VI. Chloride quantities of the order  $0.3$ – $0.4$  mg. chlorine (corresponding to the amount in  $0.1$  ml. plasma) can be determined with a coefficient of variation of  $0.5\%$ .

Over the range  $35$ – $7\gamma$  chlorine an accurate determination of the liberated iodine is very conveniently carried out by a direct colorimetric method. This may be done by the use of the Pulfrich photometer or by the 'grey solution' of Thiel and the dipping colorimeter of Leitz, provided with light filters. Messrs. Leitz now supply the dry powder for making up the grey solution, and a special glass for checking the strength from time to time. The 'grey solution' has an extinction coefficient of  $0.500 \pm 0.005$  for nearly the whole of the visible spectrum, and in its present form the grounds for previous objections have been removed.

Below  $7\gamma$  chlorine the iodine liberated in the central chamber is determined colorimetrically after adding  $0.5$  ml. of  $0.2\%$  starch. This colorimetric method with starch may be used for the whole range below  $35\gamma$  chlorine. It is then necessary to use

2 ml. 0.2% starch. An ordinary colorimeter without filters may be used.

**Procedure.**

(a) **Chloride determination down to 35 $\gamma$  chlorine.** Into the central chamber of a Unit is run approximately 1 ml. of a 20% potassium iodide solution. Into the outer chamber is introduced about 0.2 g. of pure potassium permanganate, taking care that none of the grains are spilled into the central chamber; this may be done without any difficulty when using a spoon spatula. The quantity of permanganate need be only very roughly judged and weighed but once to form an idea of the bulk. 1 ml. of the fluid to be analysed is introduced from an Ostwald pipette into the outer chamber. One of the square glass lids for covering the Unit is now lightly smeared over its surface with the special fixative (see below). The lid is placed so as to just allow the introduction of the tip of a pipette. The Unit is slightly tilted—this most conveniently by resting it on another lid—and 1 ml. of a sulphuric acid solution containing 24–30% by volume of pure sulphuric acid (s.g. 1.84) is run into the outer chamber. The lid is immediately fixed in position and the fluid in the outer chamber rotated gently about 10 times to ensure adequate mixing. The Unit is then left aside on the bench after being first examined to see if it is sealed all around—which is evident by the transparency of the contact. After 1½ hours the lid is removed and the contents of the central chamber are titrated with 0.05*N* thiosulphate from the burette previously described, using a drop of starch indicator towards the end. The iodine starch colour is purple or violet in the presence of the iodide. For this titration the Bang 2 ml. standard burette may also be used containing 0.005*N* thiosulphate.

A blank determination with the reagents is carried out at the same time.

*Calculation of the result.* After subtracting the blank value, the amount of thiosulphate is multiplied by its chlorine equivalent for the determination. This latter may be determined with fresh thiosulphate solution by making an initial duplicate determination of 1 ml. of 0.0141*N* HCl, each ml. of

which contains 0.5 mg. of chloride as chlorine. The burette reading here multiplied by 2 gives the figure required for 1 mg. of chlorine. In the special burette 0.05*N* thiosulphate is used, and when made up with a trace of sodium carbonate keeps for a long period. (See reagents below.)

If the thiosulphate is directly standardised by titration of standard iodine in 20% KI, the result, calculated as chloride, is multiplied by 1.03. This factor does not arise from the oxidation and absorption of the chlorine not being quantitative, but is due to a slight diffusion of the liberated iodine from the central chamber. The factor 1.03 applies for  $1\frac{1}{2}$ – $2\frac{1}{2}$  hours after the addition of the sulphuric acid to the outer chamber. (This point is again referred to below.)

The above method is independent of the presence of protein to the extent of 1 mg., i.e. 0.1% in 1 ml.

If it is found desirable to carry out a determination with greater or lesser volumes than 1 ml., the total acidity in the outer chamber should lie between 12 to 15 volumes % of sulphuric acid. This and other conditions are discussed subsequently.

If there is not much organic matter present it may be found more convenient to add the permanganate as 1 ml. of the saturated solution to 1 ml. of the solution for analysis in the outer chamber. The acid subsequently added should be 0.5 ml. of 60–75% (by volume) sulphuric acid.

#### Reagents.

1. 20% *potassium iodide*. This solution does not require to be accurately made up. It should be stored in a brown bottle and is best made in small quantities at a time. A small amount of free iodine will not affect the result since it will be allowed for in the blank. For chloride determinations below 35% chlorine the potassium iodide should, however, be freshly made up, or at least give no perceptible colour when viewed in a thick layer.

2. 24–30% (*by volume*) *sulphuric acid*. The purest sulphuric acid should be used with s.g. 1.84, 24–30 ml. being run into about 60 ml. distilled water in a beaker and made up to 100 ml. after cooling.

3. 0.05*N* *thiosulphate*. This is made up in the usual way by

dissolving 12.42 g. to make 1 litre of solution. To preserve the solution 50 mg. of sodium carbonate are added per litre.

4. *Potassium permanganate.* Only the purest analytical reagent should be used. The crystals are ground to a powder in a clean mortar and preserved for convenience in a small wide-necked stoppered bottle.

5. *The fixative.* This is formed by melting 50 g. of paraffin wax (M.P. 49° C.) in 80 ml. of pure liquid paraffin in a beaker and cooling the mixture; more or less of solid paraffin may be used in accordance with the prevailing temperature of the room. The paraffin wax supplied by the British Drug Houses is suitable for use, though some samples supplied at higher melting points were not sufficiently free from impurities, when carrying out the finest determinations.

6. *0.2% starch solution.* The most efficient of such solutions found by the author is that of Mutnianski as described by Kolthoff (115). In this method 2 g. of soluble starch and 10 mg. mercuric iodide are ground to a paste with a little water and poured into 1 litre of boiling water. The solution is clear and does not alter over a long period if kept in a brown bottle.

**Accuracy, etc., of method.** The coefficient of variation is 0.55%. In a typical group of 25 determinations 23 gave results within 1.2% of the theoretical.

It may be noted that the method can deal, practically speaking, with the largest amounts of chloride, but at the same time—as above mentioned—it is advisable for convenience in titration that the fluid be diluted if necessary, so that 1 ml. contains not more than about 0.7 mg. chlorine (or not more than twice the strength of a 1 in 10 blood filtrate). Smaller volumes than 1 ml. may also be used, with a slightly reduced accuracy. The total acid strength in the outer chamber, however, should always be within 12–15% by volume.

(b) **Micro-determination of chloride below 35γ chlorine.**

1. *Without standards.* By the use of more dilute thiosulphate solution the titration method can be carried much lower than 35γ chlorine. Below this level, however, a colorimetric method will be found more suitable. In this the colour of the formed

iodine may be directly examined or firstly treated with starch. By the direct examination accurate determinations may be made to 7 $\gamma$  chlorine. A yellow colour is not a suitable one for colorimetry of the ordinary kind, but by the use of a spectral filter this difficulty is removed. The visual judgment with monochromatic light becomes then a judgment between dark and light and not between depths of colour. While a standard iodine solution may be used on one side, we have found it very convenient to use the absolute colorimetry already described.

*Procedure.* In a determination, 1 ml. of 20% potassium iodide is accurately delivered into the central chamber of a specially cleaned Unit and into the outer chamber 1 ml. of the fluid for examination. The lid is smeared with the fixative and placed in position. Then in accordance with the usual procedure 1 ml. of an acid-permanganate mixture is introduced into the outer chamber. This acid-permanganate mixture is most suitably formed by mixing equal volumes of saturated permanganate and 60% (by volume) of sulphuric acid in the required quantity. (The mixed solution does not keep and should be used only on the day of mixing.) Two hours after the addition of the acid permanganate mixture the lid is removed and the iodide in the central chamber examined colorimetrically, being pipetted into a colorimeter cup or cell of the Pulfrich photometer. If the Leitz colour filters are used, filter No. 3, allowing light of wave-length in the region 464  $m\mu$  to pass, is suitable to use. The use of this filter is assumed in the calculation.

*Calculation of the result.* Where the 'grey solution' is used the depth of the plunger in the iodide from the central chamber is set at 37.0 mm., and the mm. of 'grey solution' on the other side, having a corresponding absorption, gives at once the chloride analysed as  $\gamma$  chlorine, or parts of chlorine per million.

If the light absorption is inconveniently great a simple subdivision of 37.0 is used for the fixed plunger. Thus at 35 $\gamma$  chlorine the plunger is fixed at 9.25 mm. and at 7 $\gamma$  at 37.0 mm., the result in the former case being multiplied by 4. For the

best matching it will be found in general advisable to choose the depth of the fixed plunger, so that the equivalent depth of the grey solution for light absorption does not much exceed 10 mm.

**Accuracy of method from 35 to 7 $\gamma$  chlorine.** At the upper part of this region the coefficient of variation has been found to be 1.7% and 4.3% towards the lower.

**Chloride determination below 35 $\gamma$  chlorine—using starch.** The addition of 2 ml. of 0.2% starch to the iodine in the central chamber will multiply the light absorption, using the same spectral filter, by 7. The method is described here for chloride quantities at and below 7 $\gamma$  chlorine and with only 0.5 ml. starch addition, in which case the light absorption is multiplied by 14 when compared with the direct colorimetric method. Where the whole region below 35 $\gamma$  chlorine is being investigated by this starch method it is necessary to add 2 ml. of 0.2% starch, as already mentioned.

**Procedure.** The method described in the previous section is followed down to complete absorption of the chlorine. After the two hours' absorption, 0.5 ml. of 0.2% starch is added to the fluid in the central chamber and mixed, the mixed fluids being then pipetted into a micro-colorimeter cup of 1 ml. capacity. Ordinary colorimetric methods with standards may be used. We have, however, found it somewhat more convenient to work here also with the 'grey solution'. Using monochromatic light as before (Leitz filter No. 3), Beer's law is applicable in the colorimetric analysis with starch down to 1 $\gamma$  or less of chlorine (see Fig. 33).

**Calculation.** The plunger in the starch-iodine from the unit is fixed at 25.8 mm. (or convenient submultiple) and the reading of the 'grey solution' with light of wave-length 464  $m\mu$  (Leitz filter No. 3) gives an approximate value for the chloride analysed as  $\gamma$  chlorine multiplied by 10. A blank determination must be also carried out and subtracted from this figure.

Since the starch-iodine colour is susceptible to change of conditions and varies a little with the kind of starch used, it will be necessary at least with any alteration of conditions to introduce a correction in the following manner. 0.1 ml. of a



0.02*N* stock iodine is run into 10 ml. of the 20% iodide used in the determinations. 5 ml. of the starch solution are then added and mixed. Some of the mixture is pipetted into a micro cup and the plunger in the mixture set carefully at 3.55 mm. The reading of the grey solution gives the required correction factor

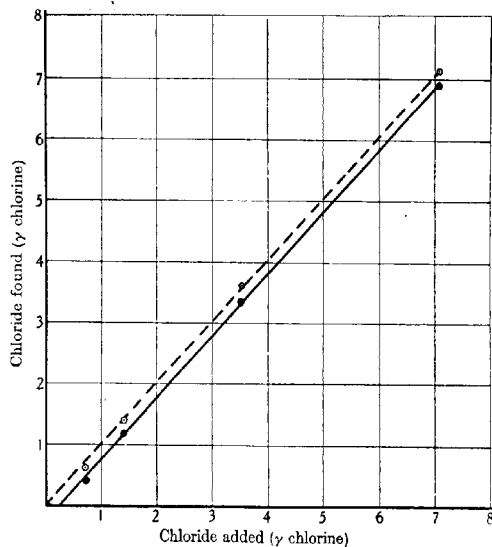


FIG. 33. — The chloride found (determined from the liberated iodine  $\times 1.03$ ), --- The result obtained using the same colorimetric method with the theoretical equivalent of free iodine added immediately to 1 ml. of 20% potassium iodide. The application of Beer's law is evident. The calculation in text allows for the factor 1.03 and the constant loss of  $0.25\gamma$  chlorine. (From the *Biochemical Journal*.)

multiplied by 10. The previous result is divided by this factor. To the result now obtained  $0.25\gamma$  chlorine is added as a constant correction. It has been found with the Unit of present dimensions and using pyrex lids with the fixative already described, that there is a constant loss of  $0.25\gamma$  chlorine. This is clear from Fig. 33. It appears that chlorine in excess of this quantity must be present before free iodine appears in the central chamber.

*Example of calculation.*

*Grey sol. mm.*  
*Filter 3*  
*(Leitz).*

Plunger at 25.8 mm. in starch and iodide mixture	
from a determination - - - - -	15.8
Plunger at 25.8 mm. in blank - - - - -	2.9
Plunger at 3.5 mm. in starch and standard iodine mixture - - - - -	10.4

$$\text{Calculation } (1.58 - 0.29) \times \frac{1}{1.04} + 0.25 = 1.49\gamma \text{ chlorine.}$$

**Accuracy of the method.** With duplicate analyses the coefficient of variation is of the order 1-2% with maximum deviations of about 0.1 $\gamma$  chlorine.

**Some notes on the methods for chloride below 35 $\gamma$  chlorine.**

(1) The Units should be cleaned very carefully and dried in a position where they are protected from dust.

(2) The use of a suitable fixative is most important, and the reader is referred to the note on the fixative under 'Reagents'; the lids are best made of pyrex glass.

(3) If the acid-permanganate is free from chloride the blank values by the direct colorimetric method will be practically indistinguishable from zero. When traces of chloride are present in stock acid and stock permanganate, allowance should be made from some initial blank determinations.

(4) The acid-permanganate must on no account be blown out, and a simple tube drawn somewhat at the end and marked at the 1 ml. (approx.) level is the most useful way of introducing the mixture.

(5) The Units during the light absorption should not be exposed to direct sunlight and are best covered with a light cloth.

(6) With the starch method the starch solution should be centrifuged for five minutes before use. With the plunger at 40 mm. in a mixture of 0.5 ml. of starch and 1 ml. of 20% potassium iodide it should require no more than 1 or 2 mm. of the grey solution for equal absorption with No. 3 filter (Leitz).

(c) **Application to chloride determination in blood, urine and tissues.**

**Chloride in blood.** The chloride is here determined in protein-free filtrates, the most suitable being the tungstate filtrate of Folin and Wu (83). Of this filtrate 1 ml. is a suitable volume giving in a subsequent titration a reading of about 20 large divisions on the burette containing 0.05*N* thiosulphate. The method described for quantities down to 35 $\gamma$  chlorine may be followed in detail. It may be found more convenient, however, to add the acid and permanganate together as 1 ml. of a mixture containing equal parts of sulphuric acid—60% by volume—and saturated permanganate. This mixture should be freshly made up in the required amount for a group of determinations, the acid and permanganate being kept separate as stock solutions. A blank determination should also be carried out with the reagents in the same dilution.

**Chloride in urine.** The chloride in urine may be determined for convenience in 0.1 ml. of undiluted urine (a slightly higher accuracy will be obtained using 1 ml. of a urine diluted one in ten). The method described for chloride determinations above 35 $\gamma$  chlorine is followed. Into the outer chamber is pipetted 0.1 ml. urine from an Ostwald pipette after the addition of approximately 0.2 g. of powdered permanganate, the acid subsequently added to the outer chamber being 2 ml. of 15% (by volume), sulphuric acid (where 1 ml. of a diluted urine is used 1 ml. of 30% sulphuric acid should be added). The remaining procedure is the same as before, the titration being carried out two hours after the addition of the acid.

**Accuracy of method with blood and urine.** The accuracy with blood is the same as that for determining the chloride in 1 ml. of 0.01*N* HCl, and may be expressed as a coefficient of variation of 0.5%.

Whereas one might have expected the chloride determinations on protein-free filtrates to proceed as with standard solutions, the large amount of organic matter in urine would at first appear likely to introduce a disturbing factor. The recovery of added chloride, however, has been found quantitative even with albuminous urine containing 1% protein. From a large

number of recoveries and duplicate determinations the coefficient of variation for the single determination using 0.1 ml. urine has been found to be 0.7%.

**Chloride in tissues.** About 0.200 g. of tissue is accurately weighed out on a watch-glass and transferred to a small mortar. For convenience of calculation the tissue weighed may lie in the range 0.190–0.210 g. A small quantity of pure quartz sand (Merck) is added to the mortar and from a graduated 2 ml. pipette or micro-burette a volume of distilled water which is calculated as follows. The weight of the tissue is multiplied by 20 and 2.36 subtracted. This with 0.2 g. tissue, for example, will mean the addition of 1.64 ml. (this volume is so chosen that the 2 ml. taken from the subsequent fluid cleared by centrifuging contain the chloride in 100 mg. tissue). The mixture is thoroughly ground to a fine paste, which should require no longer than 2 min. 2.0 ml. of 0.0667*N* sulphuric acid are added and 0.2 ml. of 10% tungstate, the mixture being further ground for half a minute. The mixture is transferred to a small centrifuge tube and centrifuged along with a similar mixture from a blank determination which will contain no tissue but 1.80 ml. distilled water and the remaining reagents as before. If it is found difficult on centrifuging to obtain a perfectly clear fluid, it is better to filter.

2.0 ml. of the clear fluid are transferred to the outer chamber of a Unit containing about 0.2 g. of powdered permanganate and 1 ml. of 20% potassium iodide in the central compartment. The determination is then made in the usual manner, 0.5 ml. of a 60% (by volume) sulphuric acid solution being used. The Units are left aside for 2 hours at room temperature and subsequently titrated or estimated colorimetrically by the method described for chloride in the range 35–7γ chlorine.

*Calculation.* Using the above method where 0.2 g. tissue is used, the total fluid volume is 4.0 ml. and the chloride corresponding to 100 mg. of tissue is contained in the 2.0 ml. of the clear fluid after centrifuging. When 0.21 and 0.19 g. of tissue are used in accordance with the above scheme it may be readily calculated that the 2 ml. of the clear fluid contain the chloride corresponding to 99.8 and 100.2 mg. of tissue respectively, which

for the purposes of the determination are equivalent to 100 mg.

The chloride equivalent of the thiosulphate used in the titration expressed as mg. chlorine gives the g. chlorine per 100 g. tissue.

Where the direct colorimetric method is used, which will be found very convenient, the plunger in the iodide is set at 9.2 mm. and the millimetres of grey solution, using Leitz filter No. 3, multiplied by 4 gives the mg. chlorine per 100 g. in the tissue.

**Accuracy of the method.** The method gives a 99% recovery of added chloride, and the variable error is similar to that already described.

(d) **Factors influencing the formation and absorption of chlorine in the above determinations.** If the conditions are varied from the above descriptions it is essential to know how changes in these conditions will affect the rate of formation and absorption of the chlorine. The factors determining these rates will therefore be very briefly discussed.

(1) *The time-absorption curve at constant temperature.* The process of oxidation by the acid permanganate takes appreciable time, so that the curve of absorption depends on two factors, namely the rate at which the chlorine is formed and the rate at which it is absorbed at unit concentration. The curve is nearly linear up to about 60% of complete absorption as shown in Fig. 34. It may be noted that the absorption of the last few % is very slow compared with the initial rate and is not explicable by the decreased concentration in the outer chamber. It is probably due to the slow release of a little dissolved chlorine from the fixative. Keeping to the conditions of the method described above, the absorption of the formed chlorine with its consequent iodine formation corresponds after 90 minutes at room temperature to 97.0–97.5% of the chloride added to the outer chamber.

Within the next hour the absorption increases slowly until all the chloride is absorbed as chlorine, but at the same time there has been a slight diffusion of iodine from the central chamber. This iodine diffusion is a constant amount of 1.2% per hour of the iodine in the central chamber. The net result

is that from  $1\frac{1}{2}$  to  $2\frac{1}{2}$  hours after the addition of the acid to the outer chamber the iodine present in the central chamber corresponds to 97.0–97.5% of the theoretical equivalent of the chloride in the analytical sample. A factor of 1.03 is therefore required in an absolute calculation of the chloride from the thiosulphate.

(2) *The effect of the permanganate concentration in the outer chamber.* This effect is shown in Fig. 35. The rate of formation and absorption of chlorine from chloride is in practically linear relationship to the permanganate concentration in the outer chamber—at least from about 2% downwards. The outer chamber in these experiments contained 0.1 ml. of 0.1N HCl, 1 ml. of permanganate solution (5, 2, 1 or 0.5%) and 1 ml. of sulphuric acid, 25% by volume (see curves D, E, F and G in Fig. 34). With one of the curves (C) solid permanganate was

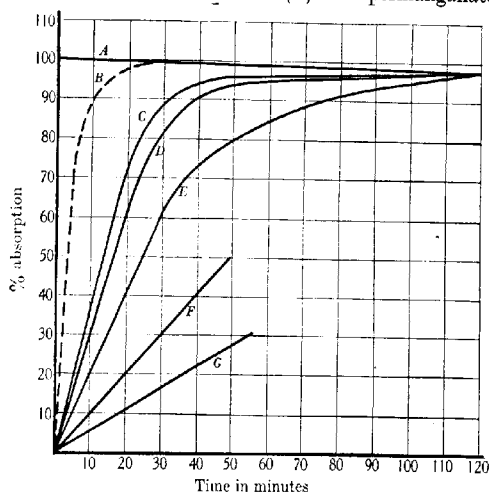


FIG. 34. Curves D, E, F and G represent the chlorine absorptions from the outer chamber containing 0.1 ml. of 0.1N HCl, 1 ml. of 25% (by volume) sulphuric acid and 1 ml. of 5, 2, 1 and 0.5%  $\text{KMnO}_4$  respectively. Curve C is similar, solid permanganate being used and 2 ml. of 12.5% (by volume) sulphuric acid. Curve B represents the absorption of chlorine from 2.1 ml. of chlorine water in outer chamber. Curve A represents the fall in percentage value of free iodine in the outer chamber introduced before a blank determination (allowance being made for blank value). Experiments conducted at room temperature of about  $18^\circ$ . (From the *Biochemical Journal*.)

used, the volume being made up to 2.1 ml. by using 2 ml. of 12.5% (by volume) sulphuric acid. The figure shows also the percentage absorption rate of chlorine (curve *B*) from 2.1 ml. of acidified chlorine water.

Where excess solid permanganate is used (about 0.2 g. added to the outer chamber) the rate of chlorine formation is but little greater than where the concentration of the permanganate in the outer chamber is 2.4%. Water saturated with potassium permanganate at 18° contains about 6%, but even with the small quantity of fluid in the outer chamber appreciable time may be taken for full saturation.

(3) *The effect of varying the acid concentration in the outer chamber.* Fig. 35 illustrates the effect of changing the acid

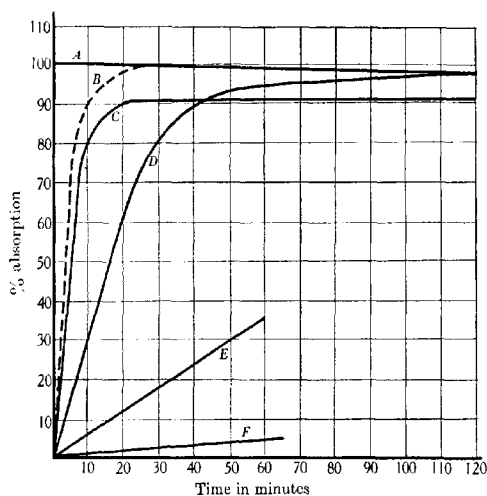


FIG. 35. Curves *C*, *D*, *E* and *F* represent the absorption rates of chlorine from the outer chamber containing 0.1 ml. of 0.1*N* HCl, 1 ml. of 5% potassium permanganate and 1 ml. of sulphuric acid of strengths 60, 25, 12.5 and 6.2% (by volume) respectively. Curves *A* and *B* are the same as in Fig. 34. (From the *Biochemical Journal*.)

concentration. The effect on the rate of formation of chlorine is here much greater than that caused by changes in the  $\text{KMnO}_4$ . The figure shows that when the acid concentration reaches 29% by volume of sulphuric acid (see curve *C*) the

absorption of the chlorine in the central chamber is very similar to that from chlorine water (curve *B*). At the same time a fall occurs in the total quantity absorbed. Below about 15% sulphuric acid this effect becomes negligible—the theoretical equivalent of iodine to added chloride being present in the inner chamber after two hours (see curve *D*), allowing for the small quantity of iodine diffused during this period from the 20% potassium iodide.

It may be noted that for such chloride determinations as here described, many other 'redox' systems with high characteristic potentials were also tried, but the acid permanganate was found the most suitable.

(4) *The effect of the fluid volume in the outer chamber.* It was shown for ammonia analyses as described in Chapter III that the percentage absorption rate of a given quantity of ammonia, other things being equal, is inversely proportional to the volume of the fluid in which it is contained. After the addition of alkali to the outer chamber all the ammonia is immediately liberated as gas, its tension being in inverse ratio to the fluid volume. With the chloride determinations, however, the absorption rate is much faster than the rate of formation with the acid permanganate concentrations used, and the amount of chlorine formed from a given quantity of chloride, under similar conditions, is independent of the volume in which it is contained. Consequently the absorption rate of chlorine is practically independent of the fluid volume in the outer chamber. However, it is found that when the volume exceeds about 3 ml. the last traces of the chlorine are more slowly delivered so that it is advisable to keep the volume below this figure.

(5) *The effect of temperature.* The determinations previously described were conducted at an average room temperature of about 18°. Judging from a few experiments where the temperature was increased to 36°, it would appear that there is an increase in the velocity of the whole process of 3 or 4% per degree rise in temperature.

The time for a determination could therefore be halved by conducting the process at 38° instead of at 18°. The fixative



described above, however, cannot be employed for this purpose as it becomes too fluid at such raised temperatures.

On account of the small temperature effect the time for a determination as given above need not be altered if the temperature of the room falls to 14 or 15°.

(6) *Effect of changes in the dimensions of the absorbing apparatus.* The above times and quantities are for the present standard apparatus. If apparatus of different dimensions is used, and we are dealing with chlorine water, the same principles would hold as considered for ammonia absorption, namely that the absorption rate is proportional to the geometric mean of the surfaces and inversely to the mean distance between. Where, however, the chlorine is formed with comparative slowness as in the above determinations this rate of formation will largely determine the absorption rate, which will be therefore independent to a certain extent of changes in the diffusing distances and surfaces.

## CHAPTER XX

### BROMIDE

**Principle and Introduction.** The principle and general technique of the chloride determinations may also be applied to that of other halogens. Both free bromine and iodine are sufficiently volatile to require about the same absorption times as chlorine. Following the micro-bromide technique described in the present chapter bromide can be determined to an ultimate standard deviation of  $0.08\gamma$  Br, which in milli-equiv. is about one-third that found with chloride. The standard deviation of the blank value may be expressed as  $0.037\gamma$  Br, which is only about one-seventh of the equivalent value found with chloride. The difference probably arises from minute traces of chloride likely to be present in water and on glassware. These ultimate errors for chloride and bromide determinations appear to be the lowest yet recorded. The early papers on micro-determination of bromide have been reviewed by Bernhardt and Ucko (116). Accounts of the more recent developments may be found in the papers of Quastel and Yates (117), Dixon (118), Bertram (119), Stoll and Brenken (120), Indovina (121), Leipert (122) and Ucko (123). Here reference will be made only to such previous work having immediate bearing on the present method and the results obtained for normal blood and urine.

Such micro-bromide determinations consist essentially of three stages. Firstly, the ashing of the material. Secondly, the oxidation of the bromide to bromine by a suitable oxidant. (These two stages may be combined in wet ashing methods, such as that of Leipert and Watzlawek (124).) Thirdly, the determination of the liberated bromine after extraction, aeration, etc.

In the present method these three stages are also followed for blood and urine, ashing being obviously unnecessary for purely inorganic mixtures or such as contain only traces of organic matter (e.g. sea water). The stages, however, are here carried

out in a special way and permit the least absolute error of bromide with the least manipulation. Also, it is possible with this method to carry out large numbers of determinations at the same time.

Into the outer chambers of the Units is introduced a dichromate oxidation mixture and in the centre 1 ml. of 20% KI in which the formed bromine is absorbed, liberating its equivalent of free iodine.

**The ashing procedure.** The ashing of the blood is carried out after preliminary removal of the proteins with methyl alcohol and subsequent evaporation of the cleared fluid. This reduces the incineration time to a few minutes and was introduced here for the reason that it is now practically certain that all the bromide in normal blood is present in inorganic form. If a complete ashing procedure is desired, the technique of Indovina (121) seems very suitable. In this method magnesium oxide is employed to prevent bromide losses on heating.

**The oxidation procedure.** The oxidant found most suitable for bromide determinations is dichromate and sulphuric acid. The dichromate is used in powdered form and the sulphuric acid as 1 ml. of a solution containing 40 vol. % of sulphuric acid to 1 ml. of the ash extract. (Upwards of 48 vol. % may be used after bromide administration.) Acid dichromate of this strength has a scarcely detectable influence on 1 ml. of 1% NaCl even after 20 hours' action, whereas all the bromide is oxidised and absorbed in 2-2½ hours.

The presence of iodide interferes with this method, as shown in the original account. With normal blood and urine this is of little or no importance as the error introduced will not exceed 1%.

After administration of iodide, the iodide present may be removed as iodine by treating the ashed extract in the outer chamber with dichromate in about 0.1N sulphuric acid, the inner chamber being empty. Raising the acidity to the required degree will then liberate the bromine, which is absorbed and determined as usual. Instead of letting the formed iodine escape, it may be determined in a similar manner to bromide.

The use of a suitable acid permanganate mixture would have the advantage of rendering the method independent of the presence of both chloride and iodide. It was found, however, to have the disadvantage of causing marked losses as bromate, when the concentration of bromide for determination is very small.

**The determination of the bromine formed on oxidation.**

In the present method the bromine diffuses into the central chamber of the Unit, liberating its iodine equivalent from 20% KI. The iodine is then determined as for chloride by titrating with thiosulphate if more than 80  $\gamma$  Br are present; an absolute colorimetric determination of the iodine colour itself is likewise found very suitable for the range from 80 to 16  $\gamma$  Br. Below this level, starch is added and the colour produced is colorimetrically determined.

In the method used by Leipert and Watzlawek (124) for the determination of the bromide content of normal blood the formed bromine is oxidised to bromate after absorption into alkali, the bromate being then determined iodimetrically. An advantage of this procedure is that six times the equivalent of the original bromine is finally present as iodine. This variation could also be used with the Unit, with possible advantage for the minutest bromide determinations.

The delicacy of the method selected, however, is so great that it is doubtful if a substantial increase in accuracy would result, and further operations with numerous reagents would be required. Also errors in the bromine formation and absorption are likewise multiplied by six.

With the present method, using starch, each  $\gamma$  Br will in fact cause an increase in the extinction coefficient of the mixed fluid from the central chamber of 0.09 (for a wave-length of 464  $m\mu$  approximately).

Concerning specific colorimetric reactions for bromine, mention may be made of the use of the reagent of Guareschi (125). This was used, for example, by Oppenheimer (126), by Bernhardt and Ucko (116) and recently by Indovina (121). The reagent is basic fuchsin decolorised by sulphurous acid and is in fact the Schiff aldehyde reagent used by Guareschi for

bromine detection. (Indovina decolorises with sulphuric acid.) It gives a violet colour with free bromine.

From Bernhardt and Ucko's account the reagent would appear to be far from satisfactory, and it may be noted that Ucko (123, 127) has abandoned it in favour of the fluorescein reagents (Swarts, ref. 128, as cited by Bernhardt and Ucko, ref. 116; Baubigny, ref. 129), which with free bromine forms eosin, or tetrabromo-fluorescein. Using this latter reagent for the determination of bromide in normal blood and tissues, Ucko (123) has obtained markedly different values from his previous results (116).

It is likely that the fluorescein reagent could be also used with the Unit, but does not seem to offer any advantage over the present method.

#### Procedure.

##### Inorganic.

(a) *Above 80% Br.* Into the outer chamber of a Unit is placed a small quantity of pure powdered potassium dichromate—roughly about 0.2 g.—by means of a spoon spatula, and into the central chamber 1 ml. of 20% KI. Into the outer chamber is now introduced 1 ml. of the bromide solution and the lid smeared with the fixative (see below) and placed in position. The Unit is slightly tilted by resting it on the edge of a spare lid, the lid is slightly displaced and 1 ml. of a solution containing 40% (by vol.) sulphuric acid is run in quickly without blowing—best from a simple tube with a mark at 1 ml. approximately. A solution containing 45% acid (by vol.) may be used when the Br/Cl ratio exceeds 1/500. After the introduction of the acid the lid is quickly replaced. The fluids in the outer chamber are well mixed by rotation. After 2 hours the free iodine in the central chamber is titrated with 0.05*N* thiosulphate from the special burette, the number of divisions on the burette being multiplied by the bromine equivalent or factor which may be determined as for chloride by making some initial determinations with standards or titrating standard iodine in 20% iodide in the central chamber. In this latter case the thiosulphate required is multiplied by 0.97 to allow for the

slight constant loss of iodine from the central chamber during a determination.

(b) *Below 80 $\gamma$  Br.* The procedure is carried out in the same way as for the previous method, except that the 1 ml. of iodide (which should have been carefully delivered) in the central chamber is examined colorimetrically. This is done either by direct colorimetric investigation without standards or by the addition of 0.2% starch solution and subsequent determination.

*Without standards (to about 16 $\gamma$  Br).* The contents of the central chamber are pipetted into a micro-colorimeter cup and the plunger set at 8.35 mm. or convenient multiple. Using the 'grey solution' of Thiel and Leitz filter No. 3, the depth in mm. of grey solution multiplied by 10 gives the  $\gamma$  Br analysed. 1.4 $\gamma$  is added to the result, being a small constant loss as bromate (Fig. 36). It is advisable that the depth of the fixed

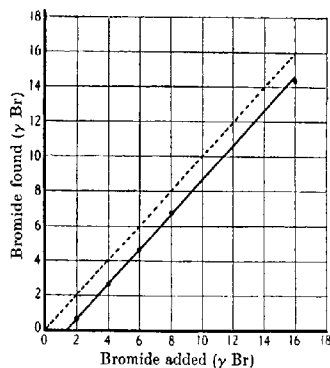


FIG. 36. — The bromide found (determined from the liberated iodine  $\times 1.03$ ). - - - - The result obtained using the same colorimetric method with the theoretical equivalent of free iodine added immediately to 1 ml. of 20% KI. The calculation in the text allows for the factor 1.03 and the constant addition of 1.4 $\gamma$  Br. (From the *Biochemical Journal*.)

plunger be so chosen that the mm. grey solution required for a match do not much exceed 10 for this direct colorimetry, though this precaution is not necessary when using starch. For this absolute colorimetry the Pulfrich photometer may also be employed or the new Leifo instrument of Leitz, the extinction

coefficient being given directly on the instrument and the bromide concentration being subsequently read from a graph or tables.

*With starch addition (to about 2γ Br).* This method is most suitable for bromine quantities under 16γ, but may be used to cover the whole range from 80γ Br downwards. Where the amount is under 16γ, 0.5 ml. of a 0.2% starch solution is added to the central chamber, but for the range between 80 and 16γ, 2.0 ml. of the 0.2% starch solution must be used. The mixed contents from the central chamber are pipetted into a micro-colorimeter cup and the iodine is estimated by comparing it with standard iodine in 20% KI similarly treated, or by absolute colorimetry. Using the grey solution, the fixed plunger in the starch-iodide solution is put at 29.0 mm. (or convenient multiple or submultiple) and the reading of the grey solution multiplied by 0.2 with subsequent addition of 1.4 gives the γ bromine analysed. Leitz filter No. 3 (464 mμ) is used. (If 2 ml. starch have been added the reading is multiplied by 0.4.) The values obtained by absolute colorimetry may be checked by standard iodine and starch as described in the chloride method. It may be mentioned, however, that with absolute colorimetry in relation to the starch-iodide colour a constancy is obtained from day to day using the same starch solution. The whole procedure below 80γ Br may then be conducted without standards.

#### **Ashing and extraction procedure for blood and urine.**

**Normal blood (1 ml.).** Into a 15 ml. centrifuge-tube is pipetted 1 ml. of blood, 12 ml. of 95% methyl alcohol are added and the whole is well mixed. The tube is then capped and centrifuged for 5 minutes. 10 ml. of the clear fluid are pipetted into a small crucible (20–30 ml. capacity) and 0.2 ml. of a standard KBr solution containing 2γ Br is added. The fluid is dried in the oven at 90–100° and then the crucible is heated in the Bunsen flame until the bottom and sides have been brought for about 1 minute to a dull red heat. It is then cooled and 0.5 ml. of distilled water is pipetted into the crucible, mixed and rotated around the walls. The solution is then pipetted into

the outer chamber of a Unit. Another 0.5 ml. water is added and the process repeated. The presence of some carbon particles does not interfere. The determination then follows the inorganic procedure (above outlined) for quantities under 16 $\gamma$  Br. The 2 $\gamma$  Br are added to bring the amount analysed on to the linear curve. A blank determination is carried out in an exactly similar manner, including the small bromide addition. The calculation of bromide analysed is then made as above, the blank value being subtracted and the addition of 1.4 $\gamma$  Br here omitted.

**Blood after bromide administration (0.2 ml.).** A similar procedure is adopted. 5 ml. of 95% methyl alcohol are used and 3 ml. of the clear fluid pipetted off for drying. No bromide need be added. The mode of determining the free iodine in the central chamber will depend on the order of magnitude of Br expected. It may be stated that the ingestion of 2 g. KBr by the human subject will produce about 16–32 $\gamma$  Br in 0.2 ml. of whole blood.

The recovery of bromide from Folin-Wu filtrates is described below.

**Urine.** The same procedure for ashing and extraction may be adopted for normal urine as for urine after bromide administration. Since the bromide in urine very closely follows the chloride it is always advisable to carry out a preliminary chloride determination. The bromide concentration has in fact little significance apart from this chloride figure. Where the urine contains the average quantity or more of chloride, 2 ml. are used for ashing with correspondingly larger volumes with reduced chloride concentrations. 2 ml. urine are pipetted into a crucible and dried in the oven. The crucible with dried contents is now held over the Bunsen flame and cautiously incinerated. The contents char at first, then fumes are emitted, the residue finally melting as a rule when the bottom of the crucible turns red. At this stage the crucible is withdrawn from the flame and cooled. The whole procedure does not require more than a few minutes, and the bottom of the crucible is brought only for about 1 minute to a dull red heat. After cooling, 2 ml. of distilled water are added and mixed with the



contents. After a short time 1 ml. is pipetted into the outer chamber of a Unit, the standard method being then followed. If it is found difficult to obtain 1 ml. free from many charred particles, 2 ml. of extract are centrifuged in a small tube, which procedure may be followed as a routine. After bromide administration, the free iodine present in the central chamber can be titrated with the  $N/20$  thiosulphate from the horizontal burette.

**Notes on method and solutions required.** No special care need be taken to free the analytical reagents used from bromide, since it is found that they contain none. But it is advisable to clean the Units, after the usual cleaning, with a little alcohol and ether, subsequently rinsing them with distilled water. For the smallest bromide quantities the Units should not be exposed to direct sunlight during the absorption, and may be covered with a light cloth; under these conditions the blank values will show no alteration even after 24 hours or more. Another point that may be emphasised is that 0.5 ml. of 0.2% starch is no longer sufficient when the bromide exceeds 20  $\gamma$  Br. 2.0 ml. are then a convenient amount and will be sufficient for the whole range.

*40% sulphuric acid.* This is made up as a volume concentration, 40 ml. of the purest sulphuric acid (sp. gr. 1.84) being made up to 100 ml. with water. A certain latitude (of a few % at most) is permissible in the actual concentration, but the purest sulphuric acid must be used. Forty-five vol. % is advisable when the Br/Cl ratio exceeds 1/500.

*20% potassium iodide.* The purest reagent should be employed, and for the minutest bromide determinations it should be freshly prepared. When over 80  $\gamma$  Br are being determined, a slight coloration is of no consequence as it will be allowed for in the blank.

*0.2% starch.* This is best prepared as described for chloride determinations in the previous chapter.

*The fixative.* This is also the same as for chloride.

*0.02N iodine.* This is made up as a stock solution from  $N$  or 0.1N iodine and stored in a brown bottle in the dark.

*Standard bromide.* 1.489 g. of pure powdered and dry KBr are dissolved in 1 litre of water. Then each ml. contains 1000  $\gamma$  Br.

**Accuracy of the method.** The accuracy for the higher quantities resembles that already found for chloride, 0.6% as a coefficient of variation being found in a large series of determinations of 800 $\gamma$  Br. For the lowest quantities the bromide method is somewhat more accurate than the chloride owing to the smaller blank values—which arise only from the light absorption by the added starch with occasional traces of free iodine in the 20% iodide. In the region 8 to 2 $\gamma$  Br, the maximum deviation to be expected with careful working is approximately 0.2 $\gamma$  Br.

The constant loss of bromide is somewhat higher than the corresponding chloride loss. As noted above, it has been found to be 1.4 $\gamma$  Br. in a large series (see also Fig. 36), but for these lowest determinations it should always be determined independently, as unsuspected slight changes may affect it. A curious result has been found by the author in this respect, namely, the addition of a small amount of potassium chromate to the dichromate (1% or less) reduces the loss to 0.5 $\gamma$  Br. The reason for this is not clear.

**The effect of chloride and iodide on the bromide determinations.** For biological determinations it is essential that chloride should have no appreciable influence since it will almost always be present in largely preponderating concentrations. With the above determinations in the 2 hours of bromide absorption the chloride oxidation is scarcely measurable when the chloride concentration in the outer chamber is of the order of 0.5–1.0% NaCl (i.e. 1–2% in the fluid examined). Even after 24 hours' action the amount represents less than 2 $\gamma$  Br.

Iodide, on the other hand, interferes and will increase the free iodine in the central chamber. It is, however, readily removable. If to 1 ml. of the fluid in the outer chamber 0.2 g. dichromate be added and 0.1 ml. of *N* sulphuric acid, then by mere exposure of the Unit on the bench—the central chamber being empty—all the iodide will have disappeared in one and a half hours, the free iodine diffusing into the atmosphere. If the iodide present much exceeds 100 $\gamma$  iodine, this exposure time should be extended.

**Note on the bromide content of normal blood and urine.**

Using the above method, values for normal blood bromide varied between 227 and 572 $\gamma$  Br/100 ml., the mean value being 372 $\gamma$ /100 ml. The corresponding urine samples gave a mean value of 656 $\gamma$  Br/100 ml. with a range of 297–855. The chloride of the urine samples was determined as well as the bromide, and the ratio of chloride to bromide (expressed as milli-equivs.) found to be 2150. The chloride in the blood samples was not determined, but if we assume that the mean ratio of bromine in the whole blood to bromine in the plasma is 1 : 1.12—a figure derived from Leipert's data (122)—and that the concentration of chloride in plasma is rather constant and 10.35 milli-equiv./100 ml., then we may derive the mean value of Cl/Br for the plasma as 2180. Comparing this value with the corresponding ratio of 2150 for urine, it appears that compared with chloride the bromide in urine, shows an almost identical ratio with that in blood. Leipert found with his method a somewhat higher bromide ratio in urine.

Pincussen and Roman (130), supported by Zondek and Bier (131), advanced the theory that normal blood bromide consisted largely of bromide combined in organic linkage and functioning like thyroxine as an important catalyst in the organism. The pituitary was thought to be the site of the formation of this substance. Its passage from the pituitary into the mid-brain was believed to be associated with the advent of normal sleep and the content of bromide in the blood to be much reduced in maniacal depressive states. The views of Pincussen and Zondek were based, however, on faulty methods as shown by Fleischacker and Scheiderer (132).

Ucko (127), who with Bernhardt (Bernhardt and Ucko, 116, 133) had obtained very high bromine values for the pituitary, has now stated that using a revised method he could not find more bromide in the pituitary than in other glands, and that no valid evidence exists for assuming the presence of an organic bromide catalyst or for the assumption that a gland is associated with its production. From the work of Quastel and Yates (117), Dixon (118) and in particular the recent detailed studies of Leipert (122), and of Leipert and Watzlawek (124), there are

no valid grounds for supposing that the normal blood bromide is other than the inorganic substance. Leipert has shown that protein precipitation, dialysis or ultrafiltration gives no indication of a bromo-protein compound. Moreover the bromine of blood is completely precipitated by silver nitrate, whereas organic bromide compounds of the type of dibromotyrosine are not precipitated by this reagent.

Dibromotyrosine is one of the two organic bromine compounds known to occur in nature, being found in corals, etc. (Mörner, ref. 134). The other is 6-6 dibromo-indigo, found to be linked with protein in *purpuridae* (Friedlander, refs. 135, 136), and which appears to be identical with the ancient Tyrian purple.

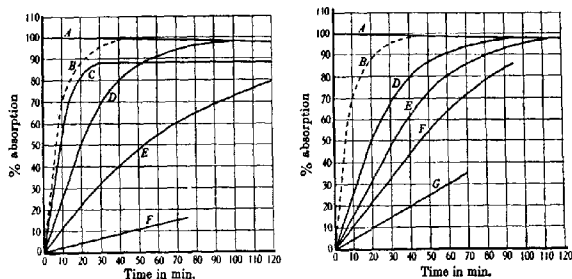
Concerning the origin of the normal blood bromide, the greater part of this must come from the bromide associated with the natural chloride of the food, although this does not often exceed 20% of the total ingested. The Br/Cl ratio in commercial chloride has been found here not to exceed 1 : 7000 for four samples, whereas the Br/Cl ratio of sea water and of ordinary drinking water (e.g. Dublin Vartry water) is of the order 1 : 700 to 1000. Presumably the natural foodstuffs possess a similar ratio to the latter with regional variations. The commercial chloride taken would therefore dilute the 'natural' ratio—the general limiting value of which may be presumed to be that of the sea.

The failure of the evidence hitherto presented for an organic bromide compound in the blood by no means removes biological interest from micro-determinations of bromide. Among other considerations, the study of the Br/Cl ratio may throw considerable light on the chloride metabolism as well as on the functioning of such organs as the kidney. From the clinical side also an easy and accurate method for carrying out micro-bromide determinations on very small quantities (finger blood after a single therapeutic dose) is of considerable interest.

**The effect of varying the conditions in the above bromide method.**

*Varying the acid concentration.* The effect is illustrated in Fig. 37, the rate of absorption from bromine water being also

given for comparison (dotted line). It will be seen that changing the acid concentration has a marked effect of a kind similar to that shown for chloride. 20 minutes after adding the 40% sulphuric acid (as described above) 50% of the bromide has



FIGS. 37 AND 38.

FIG. 37. Curves *C*, *D*, *E* and *F* represent the absorption rates of the bromine from the outer chamber containing 0.1 ml. of 0.1*N* KBr, 1 ml. of saturated dichromate and 1 ml. of sulphuric acid of strengths 75, 40, 30 and 20% (by vol.) respectively. Curve *A* represents the fall in percentage value of free iodine in the outer chamber introduced before a blank determination (allowance being made for blank values). Curve *B* represents the absorption of bromine from 2.1 ml. bromine water in the outer chamber.

FIG. 38. Curves *D*, *E*, *F* and *G* represent the bromine absorption from the outer chamber containing 0.1 ml. *N* KBr, 1 ml. of 40% (by vol.) of sulphuric acid and 1 ml. of saturated, half-, quarter- and one-sixteenth-saturated dichromate respectively. Experiments conducted at room temperature of about 18°. Curves *A* and *B* are the same as in Fig. 37.

been oxidised and absorbed. When 20% acid is added only 4–5% of the bromide is recovered in the same time. In a similar way to the action of the permanganate system on chloride, a marked deficiency appears in the total amount recovered when the concentration of acid exceeds a certain limit (see curve *C*, Fig. 37). This deficiency, however, is not evident until the concentration of acid in the mixed fluids exceeds 24%. The concentration of acid should in fact be maintained within 24–19%, since a higher concentration than 24% causes a loss in the total amount recovered and less than 19% gives an unduly slow absorption.

In Fig. 37, curve *A* shows that a slight constant loss of iodine

occurs from the central chamber (approximately 1% per hour of the total amount present).

*The effect of changing the dichromate concentration in the outer chamber.* The effect is much less than changing the concentration of acid. Similar results were obtained with the permanganate system and chloride; but whereas the rate of chloride oxidation seemed to be proportional to the permanganate concentration, the influence of varying the concentration of dichromate appears to follow a square root relation.

The effect is shown in Fig. 38. It may be stated that saturated potassium dichromate at room temperature (18°) contains 9.8 g. dichromate per 100 ml. solution.

## CHAPTER XXI

### CARBONATES, BICARBONATES AND OXIDATION RATES OF ORGANIC SUBSTANCES

FOR quantities of carbon dioxide of the order of 1.0 mg. and bound as carbonate or bicarbonate, the method to be described, as worked out in conjunction with Dr. Isaccson, gives an accuracy of about 0.5% as a coefficient of variation. It is suitable for determinations down to 0.2 mg. or to 0.1 c.c. of the gas at N.T.P., 0.2 mg. carbon dioxide corresponding to 0.9 ml. of *N*/100 acid. Below this figure the carbon dioxide of the air begins to interfere very appreciably unless suitable modifications of the method are introduced.

The method is particularly suitable for the determination of the bicarbonate content of the blood ('alkali reserve' of Van Slyke, ref. 137), and also for the study of the oxidation rates of organic substances *in vitro*.

The carbon dioxide in the method is measured as ml. of a standard hydrochloric acid, and the titration figure can be converted at once into c.c. of carbon dioxide at N.T.P. (if required) by a single factor without any further corrections.

**Principle of method.** The total carbon dioxide is set free by acidification in the outer chamber and the free gas diffuses into barium hydroxide solution in the central chamber, where it precipitates an equivalent amount of barium carbonate.

The content of the central chamber is titrated after absorption subsequent to the addition of a drop of thymolphthalein solution, which can alternatively be incorporated into the alkali at the beginning. The thymolphthalein changes from blue to colourless at a *pH* of 9.3 (approx.).

**Procedure.** The following procedure is applicable in general to bicarbonate and carbonate solutions with carbon dioxide range of about 1.0 to 0.2 mg. Into the outer chamber of a unit

is run about 0.2 ml. of 1.0*N* sulphuric acid. This is delivered without blowing, which must be strictly avoided at every stage. It is better to have the acid at one part than spread over the whole surface. A lid is now smeared with vaseline and placed in a ready position when 1.0 ml. of the  $\text{Ba}(\text{OH})_2$  solution is run into the central chamber from a 2 ml. Bang burette with soda-lime guard (Fig. 6). It may also be delivered from a long tube pipette with 1 ml. mark. This is filled with the tip at the bottom of some barium hydroxide solution contained in a narrow test-tube, into which it is run from a stock bottle. The delivery into the Unit should be quickly carried out and the lid placed immediately in position. The Unit is now tilted by resting on a spare lid, the lid slightly displaced to allow the introduction of the tip of a pipette and 1, 2 or 3 ml. fluid for examination quickly run in, the lid being immediately replaced. These deliveries may be made very suitably from ordinary volumetric pipettes with the point of the tip broken off, and at the end of the delivery the tip of the pipette should touch the glass floor of the Unit momentarily. The pipette is standardised for such delivery.

The contents of the chamber are now mixed by rotation and the Unit left aside for 30, 45 or 60 minutes at room temperature. The burette is then accurately adjusted, the lid of the Unit removed and the fluid in the central chamber titrated as quickly as is compatible with accuracy. The end-point is a change from blue to colourless, the thymolphthalein indicator being incorporated in the alkali.

With regard to the accuracy of the rapid deliveries into the outer chamber, as described above, these can be carried out with coefficients of variation of about 0.3–0.4%, as already shown in Chapter V, when the delivery tip of the pipette touches the glass.

*Calculation.* The titration difference between the full Unit and the blank in large divisions on the burette multiplied by 0.02 gives the c.c. of carbon dioxide at N.T.P., which figure divided by the volume of fluid analysed and multiplied by 100 gives the vols. carbon dioxide per cent. The calculation is independent of temperature and barometric pressure.



**Note on the effective sealing of the Units.** For effective sealing of the Units against entry of the atmospheric carbon dioxide, the contact of lid and Unit must appear to form a circular transparent ring when looked at from above. Where the contact is not good this ring will be broken by an opaque patch. A small proportion of Units and lids as at present manufactured will not give an adequate contact for this determination and must be looked for on setting up the required number. If found they must be rejected.

**Accuracy of determination.** Where care is taken to fill the Unit and to titrate at the end in a uniform time, the standard deviation of the single determination need not exceed 0.6%. This accuracy was worked out using 2 ml. 1% lactic acid to liberate the  $\text{CO}_2$ , and may be considered to apply to the sulphuric acid procedure described. It is to be emphasised that the alkali surface must be exposed as little as possible to the air. It may also be mentioned that from the principles of Chapter IV practically the whole of the absorption time with volatile substances (such as carbon dioxide) is taken up by liquid diffusion from the outer chamber.

Consequently, a considerable diminution in the surface of the internal chamber without appreciably extending the absorption time would seem theoretically possible. This would result in a gain in the accuracy of the titration procedure, and a special Unit is being considered for such a purpose. Alternatively—for the highest accuracy—an aliquot part such as 0.5 ml. could be pipetted into a small tube and titrated as in Fig. 39, using a stream of carbon dioxide free air.

*Solutions required.*

**Hydrochloric acid.** 0.179*N*. This is made by making 35.9 ml. of *N*/1 HCl to 200 ml. with distilled water. Each large division on burette (0.01 ml.) is the equivalent of 0.02 c.c. carbon dioxide at N.T.P. or to 0.0395 mg. carbon dioxide. Once made up, this solution is permanent, and since it alone is referred to in the calculation the form of this latter is always fixed without further correction of temperature, barometric pressure, change of titre, etc. The slight alterations of fluid volume with temperature lie within the range of error.

*Barium hydroxide with indicator.* 44.9 ml.  $N/5$  barium hydroxide plus 5.0 ml. 0.1% thymolphthalein in alcohol are made up to 100 ml. with distilled water. Each ml. is the equivalent of 50 large divisions (0.50 ml.) on the burette.

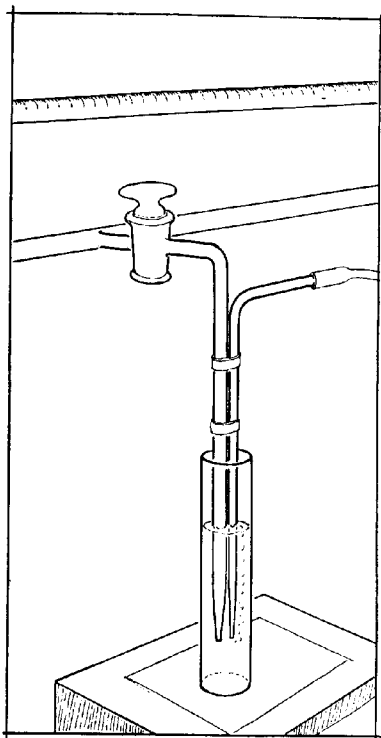


FIG. 39. Titration with the horizontal burette in a stream of carbon dioxide free air.

*Standard sodium carbonate solution.*  $N/20$  sodium carbonate solution will be found convenient (0.265 g. anhydrous sodium carb./100 ml.). Each ml. is the equivalent of 27.8 large divisions on the burette. This solution may be used occasionally as a check or control.

**Determination of the total carbon dioxide or of the bicar-**

bonate content of blood (so called 'alkali reserve'). It is essential for any true picture of the conditions that the blood be collected anaerobically. With regard to the collection of blood, centrifuging and equilibrating the centrifuged plasma with alveolar air, Peters and Van Slyke write: "The procedure gave practical information of importance in the detection of the presence and extent of disturbance of the acid-base balance. However, it does not measure directly the concentration of either bicarbonate or total carbon dioxide in the circulating blood. For this reason it has been replaced by the simpler procedure of determining the  $\text{CO}_2$  content of the true plasma or serum from blood which has been drawn and centrifuged with precautions to prevent loss of  $\text{CO}_2$ " (138).

**Collection of the blood anaerobically.** The simplest way would appear to be a collection direct from the vein (or artery in the experimental animal) through a tube dipping under paraffin oil in a centrifuge tube lined with paraffin wax and containing a particle of heparin. The blood is then immediately sealed off with some molten paraffin wax (M.P. 45) cooled on ice and centrifuged. A neat method of sealing due to Dr. Kane is used here as a routine and is of value when immediate determinations are not carried out. The liquid paraffin is sealed with a layer of molten paraffin (M.P. 45° or less), and in this is embedded a somewhat flattened wire spiral. The tube is cooled on ice and centrifuged, and in this way may be stored for days in the refrigerator. For analysis the tube is dipped momentarily in water at about 45°, then the seal is pulled out, using the spiral as a handle. The content of  $\text{CO}_2$  in 1 ml. is determined as in the method described above.

**Calculation.** The number of large divisions on the burette corresponding to the  $\text{CO}_2$  absorbed is multiplied by 2.0, giving the volumes  $\text{CO}_2/100$  ml.

**Variations of the blood  $\text{CO}_2$  in health and disease.** These are shown in the following table (Table XV), from the data assembled by Peters and Van Slyke (138).

**Note on the term 'alkali reserve'.** The term 'alkali reserve' is somewhat of a misnomer, since the bicarbonate anion represents only a fraction of the total blood buffering,

which in turn is only one-sixth the total buffering of the body at the normal or viable levels, and it would seem obviously preferable to state the result as plasma bicarbonate.

TABLE XV

	Plasma.		Arterial blood CO <sub>2</sub> vols. %.	Venous blood CO <sub>2</sub> vols. %.
	CO <sub>2</sub> vols. %.	Bicarbonate content (as vols. CO <sub>2</sub> %).		
Normal resting subject - - -	73-55	69-51	53-43	60-49
Mild acidosis, no pronounced symptoms -	55-40	51-38	—	—
Moderate to severe acidosis symptoms may be apparent -	40-30	38-27	—	—
Severe acidosis, acid intoxication - -	30	29	—	—

**Application of the foregoing method to the pH determination of circulating arterial blood.** We may write the Henderson-Hasselbalch equation for very dilute solutions in the form

$$pH = pK + \log \frac{[\text{HCO}'_3]}{[\text{H}_2\text{CO}_3]} - 0.5\sqrt{\mu}. \quad (30)$$

In this,  $[\text{HCO}'_3]$  is the bicarbonate ion concentration and  $[\text{H}_2\text{CO}_3]$  may be taken as the concentration of free carbon dioxide in solution;  $pK = -\log K = 6.33$  at  $38^\circ$ , where  $K$  is the first apparent dissociation constant at infinite dilution. (The value 6.33 increases by 0.005 for every degree centigrade below  $38^\circ$ .)

$\mu$  is the 'ionic strength' of the solution and is obtained by multiplying the concentration of each ion by the square of that ion's valence number, summing all these products and dividing the result by two (139).

For human blood plasma the equation may be written

$$\begin{aligned} pH &= 6.1 + \log \frac{[\text{HCO}'_3]}{[\text{H}_2\text{CO}_3]} \\ &= 6.1 + \log \frac{[\text{CO}_2] - [\text{H}_2\text{CO}_3]}{[\text{H}_2\text{CO}_3]}, \end{aligned} \quad (31)$$

where  $[\text{CO}_2]$  is the total carbon dioxide, and  $[\text{H}_2\text{CO}_3]$  the physically dissolved carbon dioxide. The value of  $[\text{H}_2\text{CO}_3]$  is

given as c.c./100 ml. or volume per cent. by  $\frac{100\alpha p}{760}$ , where  $\alpha$  is the Bunsen absorption coefficient at body temperature (c.c. gas dissolved in 1 c.c. at 760 mm. pressure and reduced to volume at 0°). The exact value for  $\alpha$  in serum as determined by Van Slyke, Sendroy, Hastings and Neill (140) is 0.510, so that  $[\text{H}_2\text{CO}_3]$  becomes  $0.0672p$ , and we have

$$p\text{H} = 6.10 + \log \frac{[\text{CO}_2] - 0.0672p}{0.0672p} \quad (32)$$

In this equation by inserting the total carbon dioxide of the blood (Vols. %) as determined in the preceding method for blood collected anaerobically, and for  $p$  the alveolar air carbon dioxide tension in millimetres of mercury, we get the required  $p\text{H}$  value from the equation. Thus, for example, if  $[\text{CO}_2]$  is 55.0 vols. % and  $p$  is 38.0 mm., we obtain

$$\begin{aligned} p\text{H} &= 6.10 + \log \frac{55.0 - 2.56}{2.56} \\ &= 7.42. \end{aligned}$$

(This method was used by Kane and O'Connor (141) in a study of the relation of the  $p\text{H}$  of the blood and the isodynamic law.)

**Carbon dioxide combining capacity** (similar to the older method of Van Slyke and Cullen as far as the carbon dioxide determination). This method (with the manometric analysis) is described by Peters and Van Slyke (*Quant. Clin. Chem.*, vol. ii, p. 252), as still finding a definite place when provision for the anaerobic method is lacking. It is based on the determination of the carbon dioxide content of separated plasma equilibrated with alveolar  $\text{CO}_2$  or with 5.5% carbon dioxide mixture. in a 300 ml. separatory funnel. The results are about 3 volumes per cent. too high by reason of the greater solubility of  $\text{CO}_2$  in the cold (1.6 times as high at 20° C. as at 38° C.). "The relationship is quite constant, so that for the purpose of following pathological changes in the alkali reserve in the conditions above mentioned, the  $\text{CO}_2$  capacity is entirely serviceable." It is also pointed out that in the majority of cases the physician wishes to ascertain if a diabetic or nephritic is threatened with acid intoxication or if vomiting or alkali therapy has produced

a state of alkali excess in the blood. In these conditions the plasma bicarbonate changes are gross ones, and the results similar to the anaerobic method.

The conditions in which it differs much from this latter method are those associated with over or under ventilation of carbon dioxide.

### Procedure.

*Blood collection.* The blood (5 c.c. or more) may be collected in a syringe and transferred to an oxalated centrifuge tube with the needle at the bottom of the tube, so that the blood does not fall through the air. The blood is agitated as little as possible—and only gently stirred for solution of the oxalate. It is then centrifuged and the plasma removed at the deepest level and transferred (somewhat over 2 ml.) to the separating funnel (Fig. 40). The transference need not be immediate, it being possible to store the plasma throughout the day in a clean test-tube or in a paraffined tube in the refrigerator for days.

*Saturation with 5.5% carbon dioxide.* Alveolar air may be used. The observer expires as quickly and as fully as possible after an ordinary expiration, and through the tube entering the bottle with moistened glass beads (Fig. 40). The

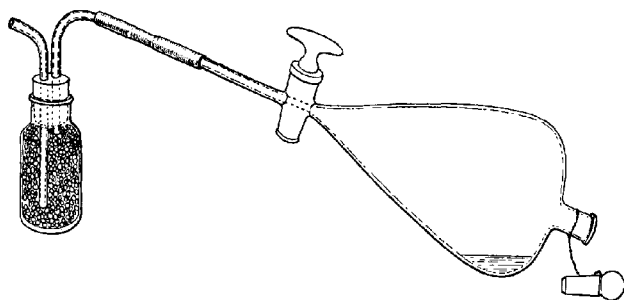


FIG. 40. Separating funnel and bottle with moistened glass beads for saturating plasma with alveolar air. (From Van Slyke and Cullen, *J. Biol. Chem.*, 1917, 30, 289.)

air leaving the glass beads will be saturated with water vapour at the temperature of the room. Immediately after the expira-

tion the stopper is inserted to prevent any back diffusion of air. The funnel is rotated for *two minutes* distributing the plasma in a thin layer over the interior. The stoppered funnel is then placed upright for a few minutes, so that the plasma drains to the lower end. One ml. plasma is removed in a long pyrex tube pipette with mark at the 1 ml. which has been standardised for delivery in 1 second or less with tip touching the glass bottom of the unit (this delivery gives an error of 0.4% as a coefficient of variation, as shown in Table III). The introduction is made into a prepared Unit which is tilted to one side, the lid opened momentarily just sufficient to introduce the tip of the pipette, which should touch the glass bottom of the unit at least momentarily after the delivery. The lid is instantly replaced and the Unit rotated, the remaining procedure being as already described. A blank determination carried out at the same time on distilled water gives an allowance both for the dissolved  $\text{CO}_2$  (not exactly, but sufficiently accurate for the purpose) and any deterioration in the  $\text{Ba}(\text{OH})_2$  solution.

**Application of the carbon dioxide method to the investigation of the oxidation rates in vitro of certain organic substances.** A number of substances of physiological importance are readily oxidisable to carbon dioxide in the outer chamber of the Unit at room temperature by oxidants of the type of acid permanganate, ceric sulphate, etc. (142). The permanganate oxidant has so far been most studied here by this method. It appears that with all such oxidising mixtures two effects on the oxidising rate may be dissociated, firstly the redox potential of the mixture, and secondly its catalysing properties. Other things being equal, an increase in redox potential increases the rate of the oxidising process, but in so far as this process involves the formation of intermediate unstable compounds, the facility with which these are formed is of very great influence on the total rate. It is possible to obtain two oxidising mixtures with exactly the same redox potential, one scarcely oxidising a certain compound when the other accomplishes this at a high rate.

## CHAPTER XXII

### QUALITATIVE TESTS FOR ACETONE AND ALCOHOL

THESE tests were elaborated by Fearon and Webb, and the following description follows closely their account in *The Analyst* (8).

#### I. The micro-detection of acetone.

Two ml. of Nessler's reagent are placed in the inner compartment of the Unit and 2 to 3 ml. of the solution under examination are placed in the outer compartment. The solution must be slightly acid in order to prevent the escape of any ammonia that may be present.

The apparatus is closed, kept at room temperature or incubated at 50° C. The presence of acetone is shown by the appearance of a pale yellow precipitate with Nessler's reagent. The method is extremely delicate. Acetone concentrations down to 0.01 per cent. give a reaction in less than a minute. At 0.002 per cent. the reaction is perceptible in about 5 minutes, and 0.0005 per cent. of acetone is detectable within an hour. When tested in this way samples of normal urines yield no precipitate even after remaining for 12 to 24 hours. If, however, a specimen of normal urine be strongly acidified with concentrated hydrochloric acid before being placed in the Unit a precipitate appears within a couple of hours when incubated at 50° C. This was found to be due to the liberation of volatile mercaptans—a contingency overlooked by some of the previous workers who used Nessler's reagent as a test for acetone in urine (143). For this reason, the urine should be acidified with a minimal quantity of dilute acid. When the solution under examination contains large quantities of acetone the precipitate in the Nessler reagent gradually redissolves in the excess of absorbed acetone vapour.



## II. The micro-detection of alcohol.

Two ml. of a solution of 2 per cent. potassium chromate in nitric acid previously diluted (1 : 2) are placed in the inner compartment and 2 ml. of the liquid under examination are placed in the outer compartment of the apparatus. The Unit is incubated at 50° C. and the presence of alcohol is shown by the development of a blue colour in the reagent as shown by W. R. Fearon and D. M. Mitchell (144).

The nitro-chromic reaction is very delicate, and with a suitable dilution of the reagent will—as shown by Webb—detect the alcohol in 0.1 ml. of a 0.025 per cent. solution after 24 hours' diffusion (29). Under these conditions, all specimens of normal urine examined showed the presence of minute quantities of alcohol.

The nitro-chromic reaction is, of course, not specific for ethyl alcohol, but responds to any compound containing the  $-\text{CH} . \text{OH}$  group. The reagent was originally shown by C. Ainsworth Mitchell to react to the presence of formaldehyde (145), and for this reason the solution to be tested for alcohol should be treated previously with an excess of 5 per cent. silver nitrate and 20 per cent. sodium hydroxide to destroy any formaldehyde that may be present.

PART III

THE ERROR OF VOLUMETRIC TITRATION

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CHAPTER XXIII

INTRODUCTORY

THE error of volumetric titration is a composite of the glass and the chemical errors, each of which is in turn divisible into a constant and a variable error.

It is necessary to define exactly what is meant by these two kinds of error—constant and variable.

We may take for example a 10 ml. pipette. Such a pipette is made to deliver 10 ml. at some standard temperature and with certain conditions of delivery and drainage. The constant error may be defined as the deviation from 10 ml. of the mean of a large number of deliveries at the standard temperature and under the stated conditions. The variable error, on the other hand, may be defined as the standard deviation of any single delivery from the mean of a large number.

The total constant error of volumetric titration will be the arithmetical sum of the constant errors of the glass ware plus the chemical error expressed in the same units. The total variable error as considered below is not the simple summation of the different variable errors.

**The manner of expressing the variable error.** With uniform temperature and mode of delivery we shall suppose that the individual deliveries from a pipette are weighed up to a large number, the mean taken and the results grouped in small regular intervals around this mean value.

If the number falling within these intervals—as percentages of the whole number observed—are plotted against the mid-point of the intervals, we shall obtain a succession of points

falling more or less closely along a special kind of curve as in Fig. 41.

This curve is described by the equation

$$y_x/y_0 = e^{-0.5 \frac{x^2}{\sigma^2}} \quad (33)$$

where  $y_x$  is any ordinate and  $y_0$  is the ordinate at  $x=0$ , at which point we may consider the position of the arithmetical mean to lie. The standard deviation is given by  $\sigma$ , and  $x$  is any deviation from the mean. The standard deviation for any distribution may be obtained by summing the squares of all the deviations from the mean, dividing by  $N$  the number of observations and extracting the square root. This may be written

$$\sigma = \sqrt{\frac{\sum x^2}{N}}. \quad (34)$$

When the number of observations is small (less than 20)  $N$  is replaced by  $(N-1)$  in this equation.

Fig. 41 is known as the *normal curve*, or the *curve of error*, and its theoretical derivation will be found described in text-books on statistics (e.g. that of Yule or Fisher, refs. 146, 147). The area under the curve in Fig. 41 may be considered to represent the total number of observations and the area under any section as the number falling within the corresponding range of deviation. Normal curves of error of the kind we are considering, distributed symmetrically around the mean value, are defined only by the standard deviation and the magnitude of the mean, but by the standard deviation only when the mean is taken as the unit of measurement.

The variable error, here defined as the standard deviation of an observation from the mean, is not confined to normal distributions, but with the latter—to which the total variable error of titration will correspond reasonably well—it is always possible to say what chances there are of an observation falling outside a given multiple of the variable error or standard deviation.

The variable error so defined has certain properties of which the following are practically important.

(1) *Variable errors are uncorrelated except in certain special instances and will add not as their sum but as the square root of the sum of their squares.* As an illustration, if we were to take the sum of two successive deliveries from a 25 ml. pipette with a variable error for the single delivery of 6.5 c.mm. the vari-

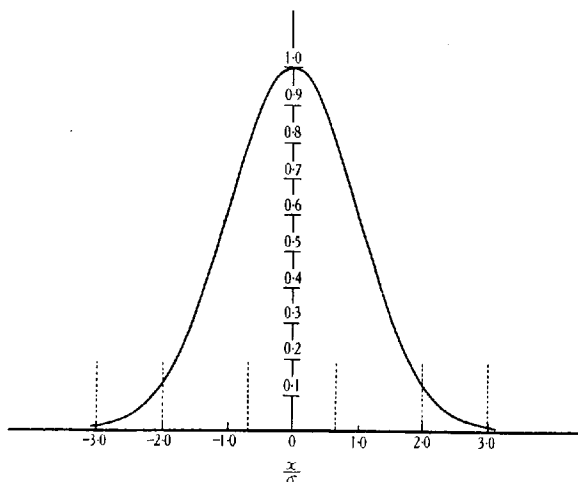


FIG. 41. The curve of 'normal' distribution. The mean is taken as zero, and  $x$  is any deviation from the mean,  $\sigma$  being the standard deviation. The first dotted lines from the zero position represent  $\frac{x}{\sigma} = \pm 0.674$  or  $x =$  the probable error. Curve from equation 33 ( $y_0 = 1.0$ ).

able error of the sum is not 13.0 c.mm. but 9.2 ( $=\sqrt{2} \times 6.5$ ). In general

$$\sigma_s^2 = \sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \dots \quad (35)$$

(For the proof of this equation for uncorrelated deviations the reader is referred to statistical text-books.)

The equation is valid for any type of distribution of the deviations, but requires that one distribution should be uncorrelated with the other, or that the chance of one deviation occurring in a particular procedure should not influence the chances of a deviation in a second procedure.

That the pipetting of one volume will not affect the distribution in the pipetting of another volume is obvious enough,

but that a pipette deviation should not influence the chances of a *special* burette deviation is not so clear, but may be presented thus. If we were to suppose that the burette delivered with complete accuracy then the distribution of its delivery on titrating would follow exactly that of the pipette—when expressed in equivalents. When, however, the burette delivers with an error of its own this latter will add quite independently of the first error and will be uncorrelated. The total error as expressed by the burette readings will then be the sum of two uncorrelated errors and their addition will be expressed by equation (35).

Similarly factors causing the variable chemical error will be uncorrelated with those causing the variable glass error.

(2) *The variable error as defined above can be treated with a precision similar to that of the constant error.*

To translate the variable error so expressed into actual limits, we may consider that the total error of titration will be sufficiently close to a normal curve of distribution that the properties of this curve can be applied. We have then the following data.

TABLE XVI

<i>Range of variable error (<math>\pm \sigma</math>).</i>	<i>% of total obs. falling outside range.</i>	<i>Chances of an obs. falling outside range.</i>
1.5	13.4	1 in 7.5
2.0	4.5	1 in 22
2.5	1.4	1 in 70
3.0	0.27	1 in 370
3.5	0.046	1 in 2,180
4.0	0.006	1 in 16,700

Plus or minus twice the standard deviation or variable error, which includes 95% of the distribution, may be taken as the practical limit of the range in titration (apart from the constant error). Three times the standard deviation may be regarded as covering all the possibilities, for it is to be noted that the curve of error here will not exactly correspond to the normal; it will be caused by a limited number of factors which are not

themselves expressible in their variability by normal curves, but rather, perhaps, by rectangular distributions.

For this reason, very small theoretical chances from the normal curve are to be taken as in fact equivalent to zero chance; and it is likely that by altering our scale of limits this has a general application in nature. One obvious rectangular distribution we shall deal with here is that of the drop or end-point emergence.

**Summation of a rectangular and a normal distribution.** This is considered as occurring around the same mean and from uncorrelated factors. Occasionally a distribution can be of a rectangular instead of a normal kind. Such a distribution of error could arise, for example, if every other error were removed in macro-titration except that arising from the size of the drop at the end-point. Over the whole range of the distribution there exists the same probability of an individual observation falling.

The standard deviation of such rectangular distributions is given by

$$\sigma_r = \frac{l}{\sqrt{12}}, \quad (36)$$

where  $l$  is the length of the rectangle. The standard deviations of these rectangular distributions add exactly as those of the normal distributions. We have again

$$\sigma_s^2 = \sigma_r^2 + \sigma_1^2 + \sigma_2^2 + \dots, \quad (37)$$

where  $\sigma_r$  is the standard deviation of the rectangular distribution. The only disability that can arise in our treatment of the rectangular distribution is that we could no longer say that 95.5% of the observations would be included in  $\pm 2 \times$  the standard deviation, though even here it may be noted that *all* observations in the rectangular distribution are included in  $\pm 1.73 \times$  the standard deviation. However, the summation of rectangular distributions of uncorrelated deviations around a mean very rapidly approaches the *normal* curve. As will be seen from Fig. 42, it is close to it after three similar summations around the mean, which in each case is represented in the

figure as 1.0. Since in actual titrations the rectangular distribution of the end-point emergence will add to the reading error and to the delivery error when the latter is relatively appreciable, as also to the pipette error, and possible variable chemical error (each of which is either a normal curve or similar thereto), it will be seen that the properties

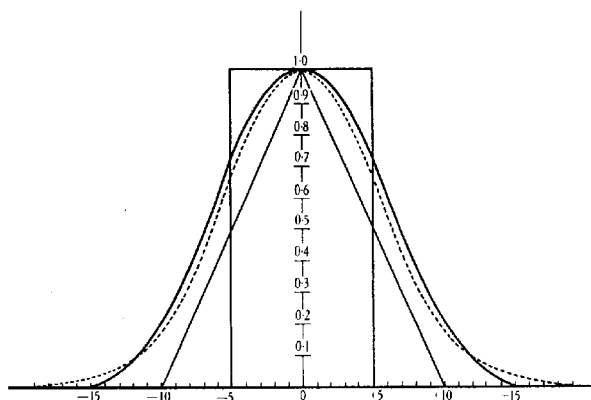


FIG. 42. Curves showing the summation of equal and uncorrelated rectangular distributions around the mean. The triangle gives the addition of two such. The dotted line is a curve of normal distribution with standard deviation a little greater than the companion curve for the summation of three rectangular distributions.

of the normal curve may be applied in general to titration results.

**Summation of the variable glass errors of pipette delivery and burette titration.** All the variable errors in the titration procedure are finally registered as burette volumes. If we had no special burette error the burette readings would follow the variations of the pipette delivery, but as equivalent volumes. If the burette solution were twice as concentrated as the pipetted solution the volume variations observed would be only half those of the pipette. In general if  $\sigma_p$  is the variable error of the pipette delivery, this will appear as a burette volume of  $\sigma_p \frac{V_b}{V_p}$ . If then  $\sigma_b$  is the special variable

error of the burette itself we have the total variable error  $\sigma_s$ , given by the equation:

$$\left(\sigma_p \frac{V_b}{V_p}\right)^2 + (\sigma_b)^2 = \sigma_s^2 \quad (38)$$

$$\text{or} \quad \left(\frac{\sigma_p}{V_p} \times 100\right)^2 + \left(\frac{\sigma_b}{V_b} \times 100\right)^2 = \left(\frac{\sigma_s}{V_b} \times 100\right)^2, \quad (39)$$

from which it appears that *the pipette and the burette errors can be added as coefficients of variation*.

Also by multiplying the equation 38 across by  $c_b^2$  ( $c_b$  = burette concentration), we obtain

$$(\sigma_p c_p)^2 + (\sigma_b c_b)^2 = (\sigma_s c_b)^2, \quad (40)$$

so that the *variable errors may also be added as equivalents*.

In a similar way the variable chemical error—which becomes appreciable when the end-point lacks definition with dilute solutions—is read as a burette volume with uncorrelated pipette and special burette errors, the three summing as equivalents or coefficients of variation.

**A numerical effect of variable errors adding as the square root of the sum of their squares.** If two variable errors are added, one of which is half the other, the addition gives

$$\sqrt{\sigma^2 + (0.5\sigma)^2} = \sqrt{1.25}\sigma^2 = 1.12\sigma, \quad (41)$$

the first error being increased only 12% by the addition. Similarly if the second error is one-third or one-fourth the first the addition gives an increase of only 5 and 3% respectively. *In the treatment of small variable errors in general, one may neglect those constituent errors the sum of which is less than one-third (or even less than one-half) the whole error.*

**Precision in the estimate of the variable error from small numbers.** So far we have been considering variable errors determined from distributions so numerous that no appreciable error occurred in the determination of the standard deviations. For the small numbers with which it is here only practicable to work—groups of 25 in general—the error in the determination of the standard deviation is appreciable. It becomes of no importance, however, when a multiple of the standard deviation or total variable error is taken in the assignment of limits, since



for such limits we may take twice or three times the variable error according to our convention. The error in the determination of the standard deviation of a normal distribution, and expressed itself as a standard deviation, is given by the equation

$$e_{\sigma} = \frac{\sigma}{\sqrt{2N}}. \quad (42)$$

For 25 results, therefore, this amounts to  $0.14\sigma$ . Hence two variable errors, determined each for 25 observations and one of which is 1.28 times the other, while very probably different (the odds being about 20 to 1 in favour), cannot be regarded as very significantly so.

*In the subsequent account, all variable errors mentioned as actual figures have been determined on distributions of 25 or some figure very close thereto, but to avoid undue repetition of the plus and minus sign and of the associated error, this has been omitted, but is to be understood as existing throughout the discussion.*

## CHAPTER XXIV

### THE VARIABLE GLASS ERROR

#### THE PIPETTE.

In delivering fluid from a pipette the chief sources of the variable error are the variation in the amount of fluid left behind on the walls, the variable adhesion of droplets to the delivery tip and the error in the adjustment of the meniscus to the mark. This last error is usually negligible compared with the others. For example, using a 2 ml. standard pipette, a range of  $\pm 1$  mm. variation of the meniscus could be allowed before producing a 10% effect on the total standard deviation of the pipette delivery. The meniscus, however, could be adjusted to within one-tenth of this range.

*Through the whole range of pipettes, therefore, the meniscus adjustment to the mark—provided this is carefully done and against a uniform background—will be regarded as a negligible factor in the variable pipette error.*

*The second error source mentioned—arising from adhesion of droplets to the delivery tip—is important in the case of micro-pipettes, and may produce a relatively large increase in the error if not provided against by a fine delivery tip or nozzle.*

**Determination of the total variable error of a pipette delivery.** The determinations recorded here have all been obtained with pipettes cleaned in the usual way by soaking overnight in strong sulphuric acid and bichromate mixture, well rinsed, and treated as a rule with absolute alcohol and ether before drying in an air current or in the oven.

The error was obtained using the pipette as if in an actual delivery for analytical purposes, the filling being done by sucking and the volume controlled to the mark by the index finger. For efficient control of the meniscus it may be men-

tioned that the finger should be slightly moist, neither very dry nor definitely wet.

The fluid was sucked to a little above the mark, the pipette tip removed from the fluid and the meniscus allowed to descend slowly to the line—against a uniform background with the eye at the meniscus level. The tip of the pipette was then touched against the glass wall of the vessel containing the stock fluid, and the pipette quickly turned into the horizontal position, the outer glass surface at the end of the pipette being cleaned with a little clean filter paper. The fluid in the pipette was then allowed to run free into a large tared weighing bottle, the pipette being vertically held with the tip above the fluid in bottle. The instant the flow ceased was noted and subsequently either of the following procedures was at first tried :

(a) The tip was pressed against the glass wall of the receiving vessel immediately on the cessation of delivery and the drop blown out sharply, rotating the tip in contact with the glass and immediately withdrawing.

(b) The tip was held for 15 seconds against the wall of the container, the latter being held so that it was slightly slant to the vertically held pipette.

(c) The tip immediately after delivery was held for 15 seconds just touching the fluid received into an originally dry container.

It was found that no appreciable difference exists between (a) and (b), though it is generally supposed that blowing out the drop gives a higher error—e.g. Kolthoff (148), Schlosser (149) (but not Ostwald, ref. 150)—and this no doubt is true if the drop be blown out without due regard to time and without touching the glass wall in the manner indicated. The third method gave the highest error, though Wagner has not found it so (151), and the difference is not so great but that it may be held to come within the sampling error.

As will be shown subsequently, there are definite reasons why (a) and (b) methods should give practically identical results and why the third method may be expected to give a higher value for the variable error.

The investigation of the three methods of delivery was carried out chiefly on the 25 ml. standard pipette, but since

method (a) is not inferior to (b) and has the merit of saving time, it has been adopted in the systematic studies here recorded. It has been found by the author superior to the second method when using micro-pipettes.

(1) *Method of direct weighing.* In this determination of the variable error 25 careful weighings and deliveries are carried out, and after each delivery the fluid is removed by another similar pipette and the receiving flask or weighing bottle weighed again. The determination is always made on a series carried out in succession and under conditions as nearly similar as possible. These latter include attention to uniform water temperature, particularly necessary with large pipettes. From the weights of the deliveries the standard deviation or variable error is given by

$$\sigma_p = \sqrt{\frac{\sum x^2}{N}}, \quad (43)$$

where  $\sum x^2$  is the summation of the squares of the deviations ( $x$  values) from the arithmetical mean, and  $N$  is the total number of observations. The use of  $(N - 1)$  in the denominator instead of  $N$  as is advised for small numbers is an unnecessary refinement with 25 values. The variable error where not given as a coefficient of variation (% of the mean) may be conveniently expressed in cubic millimetres, which for this purpose may be taken as equivalent to milligrams of water.

(2) *Method of duplicate differences.* This is a method specially useful with the smaller pipettes and having certain advantages. Two similar weighing bottles of rather large size (30 ml. or more) provided with stoppers are balanced exactly one on each pan of the analytical balance. A pipette delivery is then made into each in turn and the two bottles again balanced, the difference in weight being accurately noted. This difference for the 25 ml. pipette, for example, will rarely exceed 20 milligrams. A number of such observations are made and the standard deviation of the single delivery—the variable error—is determined from the formula,

$$\sigma = \sqrt{\frac{\sum x^2}{2N}}, \quad (44)$$

where  $x$  is here the difference between the members of any duplicate and  $N$  the number of duplicates. The method has the following advantages.

(a) The determination is practically independent of small temperature changes and a series may be interrupted for any length of time.

(b) It is uninfluenced by small errors in the larger weights—if such errors exist—and the differences are generally covered by the rider on the milligram scale. For demonstration of the variable error to students it is particularly illustrative.

(3) *Method of titration.* This method may be used for very small pipettes, such as the 0.1 ml. For example, successive deliveries of 0.1 ml. of  $N/1$  hydrochloric acid are made from such a pipette and titrated with  $N/10$  carbon dioxide free alkali from a standard 2 ml. Bang burette. A similar series is carried out delivering 1 ml. of  $N/10$  hydrochloric acid and titrated in the same way. The following equation then applies

$$v_{p_1}^2 - v_{p_2}^2 = v_{r_1}^2 - v_{r_2}^2, \quad (45)$$

or the difference of the squares of the coefficients of variation for the 0.1 and the 1.0 ml. pipettes is equal to the difference between the squares of the coefficients of variation for the two sets of titrations. This must be true since every other factor in the two groups of titrations is the same apart from the pipette errors. At such concentrations also the variable chemical error does not enter.

From the coefficient of variation so determined ( $v_{p_1}$ ) the standard deviation in c.mm. is obviously given by

$$\sigma_{p_1} = 10Vv_{p_1}, \quad (46)$$

where  $V$  is the mean volume of the delivery in ml.

Such a method gives the variable error of the total pipette delivery into a vessel. As mentioned above, small pipettes may show a comparatively large increase in error due to adhesions of minute droplets to the external wall of the delivery tip. This is prevented by the use of fine delivery tips.

**The variable pipette error obtained by the above methods.**

*The error with conventional delivery times.* For this the 25, the 5 and the 2 ml. standard pipettes were examined. The pipettes were of a type corresponding to the requirements of Class A test in the National Physical Laboratory, the tip being constructed so that its terminal surface is at right angles to the axis of the tube, being ground down for this purpose and forming a superior finish to the rounding off in the blowpipe flame.

The following table gives a summary of the results obtained:

TABLE XVII

<i>Pipette.</i>	<i>Delivery time sec.</i>	<i>Internal surface sq. cm.</i>	<i>Variable error c.mm. 25 obs.</i>	<i>Coefficient of variation.</i>
25 ml.	29	75	6.3	0.025
5 ml.	22	30	2.6	0.052
2 ml.	17	18	1.8	0.090

It would appear from the table that as a working rule the variable error of the standard pipettes may be written in c.mm. as

$$\sigma_p = 1.3\sqrt{V} \quad (47)$$

and the coefficient of variation

$$v_p = \frac{0.13}{\sqrt{V}}, \quad (48)$$

$V$  in both equations being expressed as millilitres. Also the variable error may be seen to go approximately as the internal surface.

**The effect of drainage on the variable error of standard pipettes.** The following table (Table XVIII) illustrates the results from three different kinds of procedure after delivery—mentioned at the beginning of the chapter.

TABLE XVIII

<i>Pipette.</i>	<i>Delivery time secs.</i>	<i>Variable error c.mm. (25 obs.).</i>	<i>Procedure after delivery.</i>
25 ml.	29	6.3	Drop blown out as described
25 ml.	29	6.4	15 seconds' drainage, tip touching wall
25 ml.	29	9.0	15 seconds' drainage, tip touching fluid

From the results in Table XVIII, it would appear that no appreciable difference exists between the variable error after blowing out the drop immediately after delivery, with rotation of tip against the glass wall, and the variable error when 15 seconds' drainage is allowed, with tip against the wall. As will appear subsequently, this similarity is only to be expected. On the contrary the 15 seconds' drainage with the tip touching the delivered fluid would appear to cause an increase in the variable error, this changing from 6.4 to 9.0 c.mm.

In Fig. 43, taken from V. Stott's book on *Volumetric Glassware* (152), the effect of drainage on the total delivery from a 100 ml. standard pipette is given for different delivery times. The figure shows that drainage time cannot be wholly ignored, that is to say, we must proceed in relation to precise times whether immediately after delivery or after some stated period. Using 15 seconds with the 25 ml. pipette the amount drained is 8 c.mm. or about 0.5 c.mm. per second. Since we are not likely to be out by more than one second—and scarcely that from one delivery to another—when we give zero drainage, proceeding sharply from the end of the delivery, it will be seen that this has no appreciable effect on the variable error of 6.5 c.mm. We are more likely to be in error by a few seconds when draining for 15 unless this is taken each time from a stop-watch.

**The effect on the variable error of altering the delivery time.**  
*The delivery time, the internal surface and the bore of the tube*

are the three chief determining influences on the amount of fluid left on the walls and in consequence on the variable error. The effect of the tube boring will be best considered when dealing

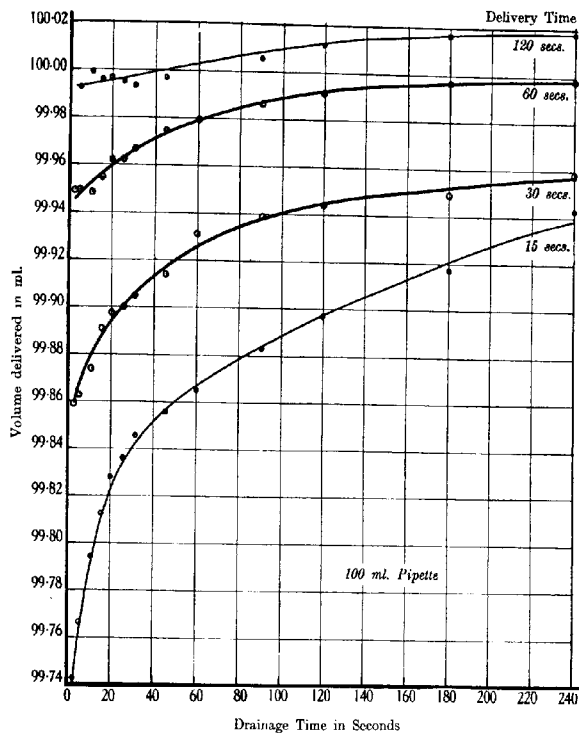


FIG. 43. Drainage curves of a standard 100 ml. pipette from Stott (*Volumetric Glassware*, Witherby, London).

with deliveries from straight tubes and consequently in the burette chapter. Before proceeding to the relation between the variable error and the fluid on the walls—the underlying relationship for glassware delivery—the general effect of altering the delivery time from pipettes may be seen from the following table.



TABLE XIX

<i>Pipette.</i>	<i>Delivery time sec.</i>	<i>Variable error c.mm.</i>	<i>Coefficient of variation.</i>
25 ml. std.	29.0	6.3	0.025
25 ml. std.	5.0	12.4	0.050
1 ml.	2.5	1.3	0.130
1 ml.	< 1.0	2.6	0.258
0.73 ml.	40.0	0.21	0.029
0.73 ml.	5.0	0.70	0.096
0.73 ml.	< 1.0	3.53	0.483

The 1 and 0.7 ml. pipettes were simple straight tube pipettes made in the laboratory. In the table is illustrated the surprising fact already commented on in Chapter V and given in Table III, that a simple straight tube 0.7 ml. pipette (length from tip to mark—16.5 cm.) can be made to deliver with the same percentage accuracy as the standard 25 ml. pipette by merely increasing the time of delivery or the rate of fall of the meniscus (as also by providing a fine delivery tip and using the blow-out procedure at end of delivery in the manner described above). The delivery time of the 25 ml. standard pipette (Table XIX) was diminished from 29 to 5 seconds by filing the tip.

**The relation between the variable error and the amount of fluid left on the walls.** *This is fundamental to the whole subject of variable error, and goes as a simple proportionality of error to wall fluid.* Provided the meniscus does not fall faster than about 15 centimetres a second (when the variable error cannot be determined with precision from the rapidity of after drainage), it is found that the variable error ( $\sigma_p$ ) and wall fluid ( $F_p$ ) are related as

$$\sigma_p = 0.05 F_p, \quad (49)$$

both expressed as c.mm.

The relation is shown in the following table (Table XX) for the 0.7 ml. straight tube pipette, the 1.0 ml. straight tube pipette and the 25 ml. standard pipette.

TABLE XX

<i>Pipette</i>	<i>Internal surface.</i>	<i>Length cm.</i>	<i>Time of delivery sec.</i>	<i>Fluid on walls c.mm.</i>	<i>Fluid per sq. cm. int. surf.</i>	<i>Variable error c.mm.</i>	<i>Ratio : Error/wall fluid.</i>
0.73 ml.	12.65	17.2	40.0	5.0	0.40	0.21	0.042
0.73 ml.	12.65	17.2	4.8	17.4	1.37	0.70	0.040
1.0 ml.	14.1	16.0	2.5	29.0	2.06	1.30	0.045
25.0 ml.	75.0	—	20.0	124.0	1.65	6.30	0.051
25.0 ml.	75.0	—	5.0	268.0	3.57	12.4	0.046

The 0.73 and 1.0 ml. are straight tube pipettes.

It appears from the table that the relation holds as closely as may be expected from 5.0 c.mm. on the wall of the 0.73 ml. pipette to 268 c.mm. on the wall of the 25 ml. pipette; or from 0.40 to 3.57 c.mm. per square centimetre internal surface.

When the delivery time is very rapid—more than 10 cm. meniscus fall per second in the simple tube pipette—the proportion of variable error to wall fluid tends to rise owing to the very rapid after drainage.

**Relation of wall fluid to delivery time.** The wall fluid was determined after delivering  $N/1$  hydrochloric acid in the usual way, taking care not to fill the pipette appreciably above the mark. Immediately after the delivery the drop of fluid in the tip was blown out as already described and the pipette then held horizontally and the external surface carefully wiped. The pipette was then rinsed five times with about one-third its volume of distilled water each time, the washings collected and titrated.

There was no difference found between normal sulphuric and normal hydrochloric acid with respect to the amount left on the walls. With one 25 ml. standard pipette five determinations with each acid gave 112 c.mm. left on the walls at room temperature ( $18.8^{\circ}\text{C.}$ ), 26 seconds being taken with this pipette for the delivery time. It may be noted that Schloesser (153) got only 0.003 ml. less delivery from a 25 ml. pipette, with  $N$  sulphuric acid than with distilled water, which amount could arise as sampling error. With the 100 ml. pipette he got 0.003 ml. sulphuric acid in excess, and with the same pipette 0.017 ml.

excess of hydrochloric acid, but all such may arise from the normal variations of the pipette delivery. One may conclude that either normal sulphuric or normal hydrochloric acids will suffice for measuring the wall fluid after deliveries.

With a 25 ml. standard pipette, in which large variations in delivery time were made either by removing small portions of the tip or by reducing the opening in the flame, the following results were obtained :

TABLE XXI

<i>Pipette.</i>	<i>Delivery time sec.</i>	<i>Wall fluid c.mm.</i>	$\sqrt{t} \times \text{wall fluid.}$
25 ml.	1.4	410	485
"	5.0	268	598
"	29.0	118	635
"	360.0	31	588

The wall fluid goes approximately in an inverse relation to the square root of the time of delivery. It will in fact be shown subsequently that with the burette or straight tubes the relation also applies, and as close as is practically required. It is not surprising here that the amount of wall fluid should have this relation with time of delivery since the fall of a viscous fluid along a surface will obey diffusion relations.

Owing to the fact that the standard pipette consists of wide barrel and narrow straight tubing, no simple relation exists connecting the wall fluid with pipette volume, but for straight tube pipettes a relation does exist between the wall fluid, the delivery time and the bore diameter. This will be considered under the delivery error of the burette.

For each single pipette type, however, the equation

$$F_w = \frac{a}{\sqrt{t}} \quad (50)$$

applies.  $F_w$  being the wall fluid after delivery, and  $a$  a constant. For the 25 ml. standard pipette  $a$  is approximately 580 c.mm.,  $t$  being expressed in seconds.

As already mentioned, there is no appreciable difference in the variable error when the drop is blown out with tip against

side rotated while blowing, and in allowing drainage for 15 seconds after delivery with tip against side. With a 25 ml. pipette it was found that the wall fluid immediately after delivery and after 15 seconds was 118 and 112 c.mm. respectively. Since the variable error goes as 5% of these values it follows that the drainage effect is here quite negligible. It would, of course, be a different matter if no attention was paid to the time after delivery and one, ten or twenty seconds taken indifferently. Whatever procedure or time be taken it must be consistently adhered to.

**The effect of temperature on the wall fluid.** Temperature affects the amount of fluid left on the walls after delivery, the relation being of an inverse kind, a fall of temperature causing increased wall fluid.

This was investigated for the 25 ml. pipette delivering normal hydrochloric acid, the temperature of the acid being varied. The following table shows the effect.

TABLE XXII

<i>Pipette.</i>	<i>Time to deliver sec.</i>	<i>Temperature of acid.</i>	<i>Wall fluid (titration corrected for density change) c.mm.</i>	<i>Wall fluid as % of whole delivery.</i>
25 ml.	24.0	31.0	94 (4)	0.376
25 ml.	24.6	18.8	111 (5)	0.444
25 ml.	24.8	11.0	124 (3)	0.496
25 ml.	—	6.5	133 (2)	0.532

(The brackets in the fourth column refer to numbers of determinations.)

No drainage time was allowed after delivering, the drop being blown out against the side in the manner already described. The effect of the temperature change from 6.5° to 31.0° may be expressed as a mean change per degree increase of -0.006% of the whole 25 ml. delivered.

Since the variable error will very probably go in the usual relation to the wall fluid we may say that temperature will have no practical effect on this since an effect of -0.0004% per degree is negligible. With regard to the constant error, however, *the effect exceeds that of the glass expansion with temperature.*

Comparison of the variable error of the standard pipettes with the tolerances allowed in construction. We may in this way compare the Class A tolerances of the National Physical Laboratory, Middlesex (and those of the Bureau of Standards, Washington), with the variable errors of the standard pipettes as in Table XXIII.

TABLE XXIII

Nominal capacity ml.	Minimum delivery time sec.	Maximum delivery time sec.	Tolerances or maximum errors allowed c.mm.	Variable error c.mm.
100	30	50	60 (80)	13
50	25	40	40 (50)	9.2
25	20	35	30 (25)	6.5
10	15	25	20 (20)	4.1
5	10	20	20 (10)	2.9
2	7	15	10 (6)	1.8

The figures in brackets refer to the tolerances of the Bureau of Standards, Washington.

It will be seen from the table that the variable error or standard deviation of the single delivery is very much less than the tolerances. Stated as in the table, however, they are not strictly comparable. Usually a plus or minus three times the standard deviation is taken as covering the range. Even multiplied by three the variable error falls appreciably below the Class A tolerances. *We are really dealing here with two kinds of error, the tolerance being a possible constant error to the limit of the value given. This possible constant error can be satisfactorily eliminated by the simple procedure of weighing five deliveries and taking the mean; for in this way we also allow for the effect of the variable error. This latter, however, cannot be removed for the single delivery under the fixed conditions in using the pipette.*

With regard to the question of standard glass error there are two points of view, that of the maker and of the laboratory worker. The maker must work to certain tolerances which are governed by the possibilities, the cost and the accuracy demand

from the laboratories. *Other things apart, the laboratory worker is constrained to work only in relation to the variable error and its implications.* Usually a certificate is provided with the best standard glassware purporting to give the exact delivery volumes under the conditions, and this bridges the two points of view for the pipette and the volumetric flask, but not for the burette, as will be shown. By determining the five delivery mean the laboratory worker can of course dispense with the certificate, and will proceed in this way for simple pipettes made in the laboratory.

**The origin and reduction of the variable pipette error.** As already shown, the variable error is proportional to the wall fluid after delivery, and it amounts to only 5% of this. It is somewhat surprising that it should be so small; and it may scarcely be profitable to inquire further into what may be its ultimate origin. There are some factors, however, we may be satisfied it is not due to. It is not due, for example, to small changes in the delivery time of the pipette leaving behind different amounts of wall fluid. To test this fourteen deliveries from a standard 25 ml. pipette were timed. The mean time was 24.8 seconds with a standard deviation of the individual time of 0.19 second or 0.8% (maximum deviation found -1.5%). Since the wall fluid goes inversely as the square root of the time, this will account only for 0.4% instead of the total 5% observed. It is also not due to small irregular vibrations imparted by the hand to the pipette since similar errors occur with tubes fixed in position, as with the burettes discussed in the next chapter. Whether superfine cleaning, such as we may expect to result from prolonged contact with hot oxidising reagents, or successive use of such and rinsing with specially prepared water, would reduce it further is a point of scarcely any practical importance, since even if it succeeded the first use of the pipette may be expected to leave it again in the condition of the ordinary well cleaned type. It may be said that it is unlikely that it would further reduce the error, since with increasing thickness of wall film due to more rapid delivery times we get a variable error going proportionally thereto.

The variable error may possibly be related to microscopic irregularities of the glass wall, and to the varying play of air currents on the fluid film as the meniscus descends.

**Reduction of error by subsequent rinsing.** When the volume conditions of the delivery are not fixed it would appear that we could remove the variable error almost entirely by subsequent rinsing. *It will necessitate about five different rinsings* and must be very carefully done, since washing above the meniscus may result in an error almost if not as great as the usual variable error.

In micro work, where we are often in the region where the variable chemical error is of the same order as the variable glass error, this rinsing may far increase the total error instead of diminishing it, owing to the increased fluid volume at the end-point.

*The objections to this washing out procedure are then that it will considerably increase the time of titration when it is carefully done, and that it may lead to a more serious error in micro work than that which it is sought to avoid.*

**Reduction of pipette error by increase of delivery time.** This is a valuable method for micro pipettes constructed in the laboratory. It will be found, where the delivery time is not borne in mind with such pipettes, that the outlet will be made too large as a rule, the pipette delivering very rapidly. With the most rapid delivery a variable error of 0.4% may be expected (vide Table III of Part I). Such an error can be reduced to 0.029 or less if the delivery time is increased to about 40 seconds for a meniscus descent of approximately 16 centimetres.

This effect is obviously of considerable importance for micro work and shows that a 1 ml. technique can be carried through with the same accuracy as the 25 ml. technique with standard glassware and without any burdensome increase in the time required. The possibility is even present of the same accuracy applying to the 0.1 ml. technique with more extended delivery times, the time required being about 2 minutes for a similar meniscus descent of 16 centimetres.

**Example of the addition of pipette errors.**

Three ml. of urine are introduced by a standard pipette into a Kjeldahl incinerating flask, and the ammonia after incineration is distilled into 25 ml. of  $N/10$  hydrochloric acid, introduced also by a standard pipette. 12.50 ml.  $N/10$  KOH are taken to titrate the acid at the end: it is required to find the variable error due to the pipetting alone.

The variable error of the 3 ml. standard pipette is 0.075% (as a coefficient of variation). The total millimols ammonia in the 3 ml. urine is  $(25.0 - 12.5) \times 0.1$  or 1.25, so that the variable error in introducing the urine is  $0.94 \times 10^{-3}$  millimols. The variable error of the 25 ml. standard pipette is 0.026%, so that in introducing 25 ml.  $N/10$  acid this amounts to  $0.65 \times 10^{-3}$  millimols. These errors add as the square root of the sum of their squares, which gives  $1.14 \times 10^{-3}$  as the addition in millimols, which when divided by 1.25 and multiplied by 100, gives 0.091%.

In a series of urine nitrogen determinations this is the variable error introduced by the pipetting of the urine and the pipetting of the acid into the receiver, using the same glassware.

When different pipettes are used each time, these must be standardised for their constant errors, and such allowed for in each series.

If we were to proceed in the above calculation simply on tolerances, our error would be computed as follows. The tolerance of the 3 ml. standard may be taken as 15 c.mm., which with the above data will mean  $6.25 \times 10^{-3}$  millimols. Similarly the 25 ml. pipette will have a tolerance of 30 c.mm., which will likewise mean  $3.0 \times 10^{-3}$  millimols. These would add directly, so that the sum would be  $9.25 \times 10^{-3}$  millimols, or 0.74%.

Working on the tolerance system means a very great and quite unnecessary increase in our computed error. It is unnecessary since the constant error—which the tolerance covers—can be eliminated to an extent negligibly small compared with the variable error, merely by determining the volume of the five delivery mean.

**Summary of the relationships of the variable pipette error.**

(a) The variable pipette error is inversely proportional to the square root of the time of delivery.

(b) It is directly proportional to the amount of wall fluid per square centimetre surface left after delivery, the relation being

$$\sigma = 0.05F.$$

(c) It is practically independent of the amount of drainage time up to the first 30 seconds or so provided the same routine times of drainage (if any) are given. Drainage in itself corre-



sponding to the ordinary periods allowed, is of no advantage in reducing the variable error.

(*d*) As shown in the next chapter, the relation of the variable error when expressed as a percentage of the whole delivery and from a straight tube is given by

$$v_p = \frac{0.022}{\sqrt{dt}},$$

where  $d$  is the diameter (cm.) and  $t$  the mean time in seconds for a meniscus fall of 1 centimetre.

With regard to the wall fluid, we have the following relations :

(*e*) The amount of wall fluid after delivery is inversely proportional to the square root of the delivery time.

(*f*) It is dependent on temperature, being decreased by 0.006% of the whole delivery per degree.

## CHAPTER XXV

### THE VARIABLE GLASS ERROR (*continued*)

#### THE BURETTE.

With the burette the significant sources of the variable error in titrating may be given as

- (1) The pure delivery error.
- (2) The manipulation error.
  - (a) The error of the end-point emergence.
  - (b) The reading error.

In addition to the delivery error, which is alone significant with the pipette, there are additional sources of error with the burette. There is the error involved in reading the meniscus level at the end-point and also the error in adjusting the meniscus to the mark which becomes appreciable with the large bore burettes. Besides which there is the error of the end-point emergence, generally taken as a drop when using the 50 ml. burette, a procedure leading to gross and unnecessary inaccuracy with the smaller burettes.

**The delivery error.** From the previous section on the pipette it may be accepted that the variable error of delivery goes proportionally to the fluid left on the walls, since this applies in general to the barrelled pipettes and to the simple straight tube kind. Here we need only consider how the amount of fluid on the walls varies with the time of delivery and with the bore of the tube, these being the two determining factors.

**Effect of delivery time on the wall fluid.** The question was investigated with the 50 ml. standard burette of internal diameter 1.11 cm.; also with a straight tube of internal diameter 0.290 cm. and with one of 0.105 cm. diameter, representing each the 25 ml., the 1 ml. and the 0.1 ml. techniques. With the simple straight tubes a piece of extra tubing was attached by a short rubber band and the end of this tube

drawn out. This was used to simulate the dead space fluid of the ordinary calibrated burette. The tube was filled from below with  $N/1$  hydrochloric acid, and when the outflowing fluid just passed below the rubber junction, the tube was quickly detached, held horizontally and the end wiped clean of external acid or any adherent drop. The tube was then rinsed to the mark with distilled water five times, and the washings titrated with  $N/200$  alkali. With the 50 ml. burette a different procedure was necessary. In this case the burette was filled from below up to the 50 ml. mark exactly and the tap closed. This acid with several rinsings was collected by inverting the burette and not through the open tap. The total fluid was titrated carefully with  $N/10$  alkali. Methyl red was used as indicator. The burette was then filled from below to the zero mark with  $N/1$  HCl and allowed to empty completely and filled again with the acid, after which the tap was fully opened

TABLE XXIV.

<i>Internal diam. 'd' cm.</i>	<i>Fluid left on walls c.mm.</i>	<i>Fluid left per sq. cm. c.mm. (q)</i>	<i>Time in sec. for meniscus descent of 1 cm. (t)</i>	$q\sqrt{t}$	$q\sqrt{\frac{t}{d}}$
I. 1.11 (50 ml. burette)	349	1.93	0.35	1.14	1.09
	234	1.29	0.72	1.09	1.04
	199	1.10	1.27	1.24	1.18
	144	0.80	2.15	1.17	1.11
	104	0.51	4.12	1.04	0.99
II. 0.290	27.3	2.05	0.110	0.68	1.26
	11.4	0.86	0.453	0.58	1.07
	7.4	0.56	0.876	0.52	0.96
	4.9	0.37	2.670	0.61	1.13
III. 0.105	14.2	1.58	0.048	0.35	1.07
	6.0	0.67	0.223	0.32	0.99
	3.1	0.35	0.630	0.28	0.86
0.105 (held horizontally)	3.0	0.33	0.845	0.30	0.93
	9.9	1.10	0.092	0.33	1.02

Length of I (50 ml. burette, graduated section) - 52.3 cm.

Length of II - 16.0 cm.

Length of III - 27.3 cm.

and 50 ml. allowed to run out, the tap being closed again with the meniscus exactly at the 50 ml. mark. The total residual acid above the closed tap was rinsed out and titrated as above.

Table XXIV gives a summary of the results obtained. It will be seen that here as with the pipette the amount of fluid left on the walls goes inversely as the square root of the time. The horizontal position for the third tube (filling done by the horizontal burette previously described) showed no appreciable difference from the vertical position.

It may be noted that a vertical burette meniscus does not descend quite uniformly throughout the whole length. Mean times with a 50 ml. burette for each 10 ml. section were as follows :

0-10 ml. in 10 secs.	30-40 ml. in 14 secs.
10-20    ,,   10 secs.	40-50    ,,   21 secs.
20-30    ,,   12 secs.	

Thus the fall is practically uniform for the upper two-thirds but falls off markedly for the last 10 ml. Using the average time for descent of 1 centimetre, no practical difference is found between the vertical and horizontal positions.

**The effect of internal diameter.** The weighted mean values of  $q$  for the three tubes where  $q$  is expressed as amount of fluid left per sq. cm. and  $t$  is the mean time for the meniscus descent of 1 cm., is 1.16, 0.60 and 0.324 c.mm. It will be seen that the bore of the tubing has a marked effect on the amount of the residual fluid. If, for example, we were to consider a thin strip of the internal surface in each tube, of the same width and extending over one centimetre along the tube's length, then the amount left on this strip with the same rate of meniscus descent is quite different for the three tubes. Obviously the difference can only relate to the tube bore. The ratio of the three means of  $q$  is as 1.0 : 0.52 : 0.28. At the same time the ratio of the square roots of the internal diameters go as 1.00 : 0.51 : 0.31. The relation of quantity of fluid, time of meniscus descent and tube bore can therefore be expressed with sufficient accuracy for our purpose by the equation

$$q = 1.1 \sqrt{\frac{d}{t}}, \quad (51)$$

where  $q$  is c.mm. of fluid per sq. cm. surface, the time in seconds for 1 cm. descent of meniscus and  $d$  the tube bore in cm.

The amount left on the internal surface of the whole tube is given by

$$\begin{aligned} q_s &= \pi dl \times 1.1 \sqrt{\frac{d}{t}} \\ &= \frac{3.45d^{1.5}l}{\sqrt{t}}. \end{aligned} \quad (52)$$

Considering the variable error, this per sq. cm. surface is given (from equations 51 and 49) by

$$\begin{aligned} \sigma &= 0.05 \times 1.1 \sqrt{\frac{d}{t}} \\ &= 0.055 \times \sqrt{\frac{d}{t}}. \end{aligned} \quad (53)$$

The most useful form of the error is here the coefficient of variation ( $v_b$ ), which we obtain by multiplying  $\sigma$  by the total internal surface, dividing by the volume in millilitres and multiplying by 100, giving

$$\begin{aligned} v_b &= \frac{0.055 \sqrt{\frac{d}{t}} \times \pi dl}{0.25\pi d^2 l \times 10^3} \times 100 \\ &= \frac{0.022}{\sqrt{dt}}. \end{aligned} \quad (54)$$

*That the coefficient of variation for the single delivery from a straight tube is given by the expression  $\frac{0.022}{\sqrt{dt}}$  is in this whole question of glass error the most significant for micro work, for by altering the meniscus descent in an inverse relation to the tube diameter the delivery error remains unchanged.*

From the foregoing section certain points of practical importance in titrating arise for comment. The total fluid left on the walls of a burette as already shown is given by

$$\begin{aligned} q_s &= \frac{3.45d^{1.5}l}{\sqrt{t}} \\ &= \frac{210}{\sqrt{t}} \text{ (for the 50 ml. burette).} \end{aligned}$$

Here  $t$  is the mean time (sec.) for a meniscus descent of 1 cm. A change of  $t$  from a mean time of 1.0 to 0.5 seconds means a change in wall fluid of 87 c.mm. or 0.18% of the full delivery, a change much greater than the delivery and manipulation errors combined. Again, if a burette is delivering 50 ml. in a mean time of 50 seconds, a change to 45 or 55 seconds means a change of about 5% of the wall fluid, approximately the same as the variable error of the delivery. Such a constant error, however, will in fact be comparatively small when we apply the usual multiple of 3 to the variable error to obtain the range. It will be found, however, that a 50 ml. burette will deliver with great constancy—to within a second or two—if the tap be fully opened, and it is obvious that this must be done in fine titration, and for such also a rough idea of the end-point position must be already known.

**The effect of drainage time on the variable delivery error.**

The effect of drainage time on subsequent rise of the meniscus has been very fully investigated by Stott (National Physical Laboratory), as given in his monograph on *Volumetric Glassware* (152). The results obtained were confirmed by Lindner and Haslwanter (154).

Drainage is very slow at first, then rises to a maximum and falls again in a wave-like occurrence. This is well shown in Fig. 44, giving the drainage rate after varying delivery times from a 50 ml. burette (scale length of 57 cm.). The crest of the wave may appear long after the delivery. This is at first surprising, but is no more than one may expect if we consider the solution on the walls as behaving like a viscous fluid.

It will be seen that drainage has no practical effect on the variable error when the burette is read immediately after the delivery. Even at the very fast rate of 18 seconds for the total delivery from a 50 ml. burette, no drainage occurs in the first 30 seconds. After this a marked drainage sets in amounting to a total of 78 c.mm. in the subsequent 90 seconds. When 40 seconds are taken for the full delivery no appreciable drainage occurs within two minutes.

As we descend into the micro scale drainage becomes more important, for not only is the absolute drainage rate increased

per unit surface, but still more so when expressed relatively. When the level of the 0.1 ml. technique is reached and using ordinary delivery times, it is necessary to use the horizontal position to avoid errors from this source, unless another device such as that of Rehberg is used. *This is perhaps the main advantage of the horizontal burette described in Chap. VI.* As an example of this increasing influence of the drainage as the bore of the burette is decreased, we may consider the drainage figures in the tables of Lindner and Haslwanter (154) for the

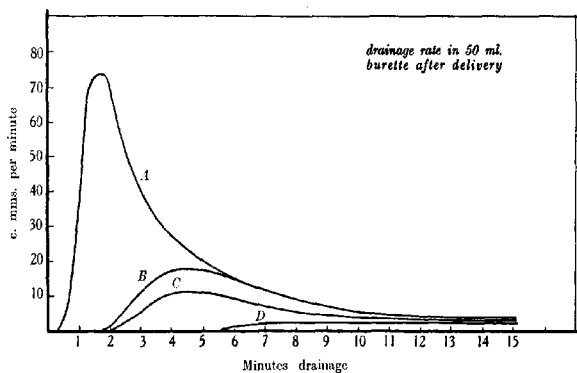


FIG. 44. Curves A-D give drainage rates from a 50 ml. burette after deliveries of 50 ml. in 18, 40, 60 and 120 seconds. Curves from data of Lindner and Haslwanter (154).

50 and 10 ml. burettes. When full delivery times are taken with the 50 ml. and 10 ml. burettes, which leave the same amount of wall fluid per unit surface, namely, 40 seconds for 50 ml., and 20 seconds for the 10 ml., the drainage after 5 minutes amounts to 0.08% and 0.387% of the total volumes respectively. Even when the same time of 40 seconds is taken for full delivery from each burette the drainage after ten minutes is 0.255% with the 10 ml. and 0.19% with the 50 ml. burette.

#### The manipulation error of the burette.

*The end-point emergence.* This for the 50 ml. burette is usually taken as a drop, which for dilute solutions and from a well-made tip should not exceed about 40 c.mm. For accurate

working with micro burettes the use of the drop as the end-point emergence must be excluded. Fractions of drops are easy to manipulate, and there will be found a rough constancy of emergence for each burette on turning the tap to secure an appreciable extra quantity of fluid. With a well-made 2 ml. Bang burette about four-tenths of a final division (4 c.mm.) is the usual minimal emergence.

With the horizontal burette previously described the least emergence that is conveniently dealt with amounts to about half a division or to 0.5 c.mm., though without a suitably fine tip this may need to be increased to one division (1 c.mm.). As already considered in the introduction to this division, the end-point emergence will represent a rectangular distribution with a standard deviation given by the equation

$$\sigma^2 = \frac{l^2}{12}.$$

For the three burettes mentioned, therefore, the end-point emergence as a variable error may be expressed by 12, 1.2 and 0.15 (or 0.30) c.mm. respectively. It is possible, of course, to work with finer emergences, but these may be described as typical of careful or even fine working.

*The reading error.* The reading error may be said to include the initial adjustment of the meniscus to the mark and the subsequent reading after the end-point emergence. It is easier to adjust a meniscus to a definite mark than to read its position between two divisions; it may be taken that this error is almost entirely due to the latter source. In the finest working the reading of the final position is brought with advantage to 0.2 of a division, but no practical advantage in accuracy would result from bringing it further.

The reading error could be treated as giving a rectangular distribution similar to the drop error, and treated as such, but since we have also the possibility of small parallax errors, plus errors due to lack of accurate discernment of the bottom of the meniscus, as well as errors in the initial adjustment of the meniscus to the mark, it is best treated as part of a manipulation error which can be determined experimentally, the manner



of determining this being given subsequent to a consideration of the meniscus reading.

*The reading of the meniscus.* In reading the meniscus the avoidance of parallax errors and the securing of a uniform back-

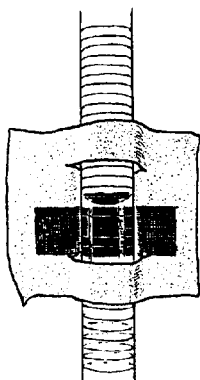


FIG. 45. Device for facilitating meniscus reading; modified from Kolthoff's '*Die Massanalyse*' (Springer, Berlin).

ground are the essentials. The former can be avoided easily by the use of burettes with the lines extending completely around the barrel. The observer places his eye so that the front and back portions of the line nearest to the meniscus coincide. With regard to the background for the meniscus reading, a simple and effective procedure is shown in Fig. 45. A suitable strip of glazed white paper is taken, a horizontal line drawn in ink and the paper inked in below it. Two horizontal incisions are then made through which

the burette is slipped in the manner indicated in the diagram. For the student it will be instructive to look at the meniscus firstly against a dull background, then to slip the paper into position from below, so that the white background just appears behind it, then to move it upwards until the black line is close to the meniscus. The effect is at first surprising.

*The determination of the total manipulation error.* The total manipulation error, made up as it is from the end-point emergence error, and the reading error, is quite independent of the total amount of fluid delivered from the burette. When 5 ml. of  $N/1$  alkali are used to titrate 25 ml. of  $N/5$  acid delivered from a standard pipette, the manipulation error of the burette—usually about 15 c.mm. for the 50 ml. burette—amounts to 0.28% as a coefficient of variation.

The pure delivery error is only about 0.02% when the meniscus descends, as usual with the 50 ml. burette, at about 1 cm. per second; the pipette error is 0.025%. These are quite negligible when compared with 0.28%, since they add as the square root of the sum of the squares. Consequently the vari-

able error of titration is here practically pure manipulation error.

Similarly the manipulation errors of the other burettes may be determined by using only a small fraction of their length. The manipulation error if so determined as a coefficient of variation, may be expressed in c.mm. as follows :

$$\sigma_m = v_b \times \frac{10^3}{100} \times V = 10Vv_b, \quad (55)$$

where

$V$  = burette volume in ml.

The manipulation errors of a 50 ml., a 2 ml. and the horizontal burette were found to be 15 c.mm., 1.3 and 0.15 respectively. *The end-point emergence constitutes much the greater part of this manipulation error and would alone account for 12, 1.2 and 0.14.*

In carrying out such manipulation tests it is essential that the burette should not be filled by pouring from above. This can give rise to a comparatively large error, since the fluid poured in runs very rapidly down the sides and after the meniscus is brought exactly to the mark will continue draining from above. It may be filled from above by a pipette or from a glass tube, or from below by means of a special attachment now common with burettes.

**The combined delivery and manipulation errors of the burette.** These errors add according to the principles outlined in the introductory chapter. *The delivery error in actual magnitude is proportional to the volume delivered, the manipulation error being independent of the volume. Stated as percentage errors the reverse is true, the delivery error being then independent of the delivered volume and the manipulation error inversely proportional to this volume.*

The manipulation error itself ( $\sigma_m$ ) may be always expressed as c.mm. (equation 55). The errors add then as

$$v_s^2 = v_d^2 + \left( \frac{\sigma_m}{10V} \right)^2, \quad (56)$$

where  $V$  is expressed as ml. and  $v_d$  is the coefficient of variation of the delivery. This is given by the relation already developed (equation 54) :

$$v_d = \frac{0.022}{\sqrt{dt}}$$

where  $d$  is the diameter of the tube and  $t$  the time in seconds for meniscus descent of 1 cm.

*The 50 ml. burette.* With the 50 ml. burette and a meniscus descent of 1 cm. per second,  $v_d$  is 0.021; the manipulation error was found to be 15 c.mm., so that it would require a delivery of 72 ml. before the delivery error as c.mm. was equal to the manipulation error. At 36 ml. delivery the delivery error is only half the manipulation error and does not much affect the total error. From 36 ml. to zero the manipulation error is dominant and the total burette error is inversely proportional to the volume delivered. At 25 ml. the total variable burette error is 0.060%, and at 10 ml. 0.15%.

*The 2 ml. Bang burette.* With the 2 ml. Bang burette with a bore diameter of approximately 0.24 cm. a meniscus descent of 1 cm. per second will give a delivery error of 0.045 as a coefficient of variation. The manipulation error was found to be 1.3 c.mm., requiring therefore a 3 ml. delivery for the delivery error as c.mm. to equal the manipulation error. Here also from 1.5 ml. upwards the manipulation error is dominant and the total variable error is inversely proportional to the delivered volume.

With a delivery volume of about 2.2 ml. the 2 ml. standard Bang burette is as accurate as the 50 ml. at the 25 ml. titration level with the conditions as stated. It would appear, therefore, that some advantage would be obtained by the use of a 3 ml. Bang burette instead of a 2 ml.

*The horizontal burette.* The horizontal burette described in text has a manipulation error, with the conditions defined in a previous section, of 0.155 c.mm.

The internal diameter is 0.105 cm., and with a delivery rate of 1 cm. per second we obtain a delivery error of 0.068% as a coefficient of variation. It would take a delivery of 0.23 ml. before the manipulation and delivery errors became equal as c.mm. The total error then as a coefficient variation would be 0.095%. *Using the burette in this way to deliver around 0.23 ml., it is the equal in accuracy of the 50 ml. burette titrating around 15 ml., the volume reduction being at the same time near to one-fiftieth.*

This presupposes, however, that the tube bore will show the same relative uniformity as that of the 50 ml. burette. When the finest grade thermometer tubing is used this degree of uniformity is secured. Also it may be said that the discriminating accuracy of the burette in titrating closely similar quantities is practically independent of bore uniformity.

## CHAPTER XXVI

### THE TOTAL VARIABLE GLASS ERROR AND ITS CONTROL

THE total variable glass error ( $v_g$ ) is given by the equation

$$v_g^2 = v_p^2 + v_b^2 + \left( \frac{\sigma_m}{10V} \right)^2 \quad (57)$$

where  $v_p$  = pipette error (coefficient of variation),

$v_b$  = delivery error of burette (coefficient of variation),

$\sigma_m$  = the manipulation error of burette in c.mm.,

$V$  = the burette volume (ml.) delivered in titration.

In considering the control of this total glass error we may take it at different volume levels. For convenience one may speak of a 25 ml., a 1 ml. and a 0.1 ml. technique, and consider each of these in turn.

**The 25 ml. technique.** We shall suppose a 50 ml. burette used with a meniscus descent of 1 cm. per second, and a manipulation error of 15 c.mm. and that 25 ml. acid are pipetted from a 25 ml. standard pipette and titrated with 25 ml. alkali from the burette. The variable error may then be computed as

$$v_g^2 = 0.025^2 + 0.022^2 + 0.060^2,$$

0.025 being the pipette error, and 0.022 the pure delivery error of the burette as considered in the previous chapter. It will be seen that the error is very largely due to the manipulation error of the burette, which would alone give 0.060%. If in the titration we were to use 45 ml. from the burette (with suitable alteration of alkali strength) the manipulation error would then give a percentage error of 0.033 and the total glass error would be 0.047%, the manipulation error being here in much lesser proportion. If we sought to reduce the total glass error further by using more than 50 ml. from the burette this would

require a refilling with the consequent errors of two meniscus adjustments. The practical procedure would consist in a reduction of the manipulation error by diminished end-point emergence, proceeding with some fraction of a drop. Proceeding with about half a drop or 20 c.mm. removed with a fine glass rod, this would reduce the total error with 25 ml. delivery from 0.07 to 0.05% and the 45 ml. delivery from 0.05 to 0.04%, the advantage in the latter case being small.

**Discrimination error.** If we were to consider a series of titrations with the same glassware and solutions, and grouped the burette readings in a succession of duplicates, then it may be shown as an elementary problem in statistics that the standard deviation of the differences between the members of the duplicates is  $\sqrt{2}\sigma$ , where  $\sigma$  is the standard deviation of the single titration from the mean of the whole series. This we may define as the discrimination error, and expressed as a percentage it may be written  $\sqrt{2}v_g$ , where  $v_g$  is the total glass error. If we made two successive titrations with the same glass and the same alkali, but two unknown acidic solutions, these could be regarded as significantly different if their titration figures differed by more than  $3 \times \sqrt{2}v_g$ . Where duplicate titrations are carried out with each acid then the range of discrimination error is  $3v_g$ , or the same as that of the total glass error for a single titration.

Using then the 25 ml. pipette for duplicate titrations and titrating 25 ml. from the burette, the discrimination range is  $3 \times 0.069$  or 0.21%, and for the 45 ml. titration it is  $3 \times 0.047$  or 0.14%. In other words, the two acids can be said to be different when they differ by more than 0.21 and 0.14% respectively. Such discrimination would be independent of the bore tolerance of the burette, since over the small volume range of such errors the bore diameter may be considered unvarying.

**Absolute error.** This involves a consideration of the constant error dealt with more fully in a later section and referred to in the chapter on the pipette. The constant glass error can be satisfactorily determined and allowed for by determining the mean weight of five deliveries and the true volume in accordance with the principles of Chapter XXIX.

As a practical exception to this easy elimination of the constant error, we have the varying bore diameter of the burette.

Burette tolerances are given in the following table :

TABLE XXV

<i>Burette ml.</i>	<i>Tolerances in c.mm.</i>		
	<i>Nat. Phys. Lab.</i>	<i>Bureau of Standards.</i>	<i>K.N.E.K.</i>
50	60	50	40
10	20	20	20
5	—	10	—
2	10	—	8

The tolerance with the N.P.L. extends to the whole or any part of the burette, but for the K.N.E.K. half the burette volume carries half the tolerance, but no further subdivision is allowed.

There would be no great difficulty, only an inconvenience, in standardising each section of the burette to find the exact divergence from the nominal value, and a burette certificate may be had in which this is already done as far as is practical. For such laboratory practice the total tolerance of the burette is comparatively unimportant, since like that of the pipette the exact volume is easily obtained, and it would seem therefore that with regard to the finest titrations an advance could be made here in a greater bore uniformity, such as will approach the variable error of the delivery.

If we standardise the burette for the whole delivery, it may be presumed that the error for any section is not greater than half the full tolerance value (60 c.mm.) existing without any standardisation. On this basis and collecting the previous data, Table XXVI has been made out.

To the range of the discriminating error given there the 30 c.mm. (half the full tolerance) has been added, as  $3.0/V$  ( $=\%$ ), the result given then as the absolute error range, or the amount which any single reading may diverge from the mean of a large number of similar titrations. It has been assumed at the same time that all constant errors have

been allowed for and that the acid and alkali are of sufficient strength to render negligible the chemical error arising from an indefinite end-point.

TABLE XXVI. *Titrations with 25-50 ml. deliveries.*

50 ml. burette. End-point emergence, 40 c.mm. Manipulation error of 15 c.mm. (standard deviation).

Pipette.	Variable error of pipette.	Volume titrated from burette ml.	Total variable glass error.	Discriminating error range. Duplicate titrations.	Absolute error range for the single titration.
25 ml.	0.025%	25	0.069%	0.21%	0.33%
25 ml.	0.025	45	0.047	0.14	0.21
5 ml.	0.058	25	0.086	0.26	0.37
5 ml.	0.058	45	0.074	0.22	0.29

**The 1 ml. technique.** The representative burette here is the 2 ml. Bang burette. We may consider firstly the magnitude of the error, using a similar procedure as with the 25 ml. technique, but at the 1 ml. level. Let us suppose a 1 ml. standard pipette is used to deliver the acid, and the meniscus descent from the burette to have a rate of 1 cm. per second. A marked difference will lie in the end-point emergence, being here a fraction of a drop or 4 c.mm., as already considered, the manipulation error being of the order of 1.3 c.mm. (st. dev.).

Considering the equation (57)

$$v_g^2 = v_p^2 + v_b^2 + \left( \frac{\sigma_m}{10V} \right)^2,$$

the usual value of  $v_p$  or coefficient of variation of the 1 ml. standard pipette is here 0.13%, and from equation 54 the pure delivery error of the burette is 0.045%, so that with the manipulation error ( $\sigma_m$ ) of 1.3 c.mm. the value of

$$v_g^2 = 0.13^2 + 0.045^2 + (0.13)^2$$

or  $v_g = 0.19.$

This is nearly three times the corresponding error of the 25 ml. technique.



**Variable titration error in an experimental series.** Before proceeding to a consideration of the manner in which this error may be reduced to that for the 25 ml. level, it is of interest to see how far equation (57) predicts the error in an actual series of titrations in which each of the three values has been experimentally determined as 0.11, 0.11 and 1.3, the rate of meniscus descent in the burette being faster than 1 cm. per second and the pipette used other than the standard type.

TABLE XXVII

<i>Acid used.</i>	<i>Variable titration error (experimental).</i>	<i>Variable titration error (theoretical).</i>	<i>No. of titrations.</i>
0.1N	0.20%	0.20%	52
0.01N	0.19	0.20	26
0.001N	0.27	0.25	20

With the 0.001N titrations the variable chemical error is beginning to influence the total error. For the 0.1 and 0.01N titrations the experimental and theoretical errors are practically identical.

**Approach in accuracy to the 25 ml. technique.** Considering the end-point emergence of the burette as 1.3 c.mm., this alone will give 0.13%, whereas the total variable error for the 25 ml. technique is 0.069% as we have seen. Refining, therefore, on the pipette or burette deliveries will be useless until either the end-point emergence is reduced further or the volume titrated from the burette is increased, in which latter case there will be a departure from a purely 1 ml. technique. The end-point emergence may without much difficulty be reduced to 2 c.mm. if the burette has been provided with a tip sufficiently fine, and burettes of such type may now be had from the makers. Alternatively the use of the Shohl (155) needle tip with Luer adapter could be considered. To the delivery end is sealed a glass Luer adapter into which fits a Luer hypodermic needle *B* of 18 to 23 gauge, cut horizontally and ground on a stone, platinum needles being obviously preferable.

With diminution of the end-point emergence to 2 c.mm. and a manipulation error then of approximately 0.7, an increased burette delivery to at least 1.5 ml. would be necessary. This would give a variable error from the burette manipulation of 0.047%. The pure delivery error of 0.045% could be allowed to remain at this level, and suitable reduction of the remaining pipette error is easily accomplished. As shown in Chapter V of Part I and in Chapter XXIV of Part III, a simple straight tube pipette delivering 0.7 ml. in 40 seconds from a straight tube pipette (16 cm. length) has an error only of 0.029%, and the error may in general be deduced from equation 54, already established in previous chapter :

$$v = \frac{0.022}{\sqrt{dt}},$$

$d$  being the bore diameter and  $t$  the time of descent of 1 cm. Assuming then a 1 ml. pipette delivering with an error of 0.029%, a burette delivery of 0.045% and a manipulation error of 7 c.mm. with 1.5 ml. titrated, from equation 57 we get a total glass error of 0.068%, which is practically identical with the error of titrating 25 ml. acid from a standard pipette with 25 ml. alkali from the 50 ml. standard burette, using a drop as end-point emergence. Nothing exceptionally difficult is introduced in this approach, the emphasis being on delivery time, fineness of tip and of end-point emergences.

**Discrimination range and absolute error.** With the technique just discussed, and which approached the 25 ml. accuracy, the discrimination range will be exactly as given for this level in Table XXVI. The absolute error range will be higher owing to a greater percentage tolerance for the smaller burette. To keep pace with the worker's possibilities here, a much reduced tolerance is required, but not in the whole burette volume, merely in the bore uniformity. The tolerance of the 2 ml. burette is 10 or 8 c.mm. On standardising the whole 2 ml. delivery it may be assumed as before that this tolerance is reduced to approximately 4 or 5 c.mm. The numerical values are assembled as before in Table XXVIII.

TABLE XXVIII. *2 ml. Bang burette. Titrations around the 1 ml. level*

Manipulation error 1.3 c.mm.—or 0.7 c.mm. in the approach to the 25 ml. accuracy—the corresponding end-point emergences being 4 and 2 c.mm. respectively. 4 c.mm. is also considered as possible error due to lack of bore uniformity after standardisation of full delivery.

<i>Pipette used.</i>	<i>Variable error of pipette.</i>	<i>Burette vol. used ml.</i>	<i>Total variable glass error.</i>	<i>Discriminating range (duplicate titrations).</i>	<i>Absolute error range of the single titration.</i>
1.0 ml.	0.13%	1.0	0.19%	0.57%	0.84%
0.7 ml.	0.029	1.5	0.068	0.20	0.47
(40 seconds to deliver)			2 c.mm. end-point emergence)		

With increasing bore uniformity in the burette the absolute error range approaches that of the discriminating range. As before, in this table the constant errors except that of bore tolerance are not considered.

**The 0.1 ml. technique.** With the horizontal burette in present use an end-point emergence of 0.5 c.mm. is secured without much difficulty, and 1 c.mm. rather easily, but in fact almost any degree of fineness in delivery can be reached by constricting the delivery tip more and by lessening the hydrostatic pressure—shortening the distance of the delivery tip from the graduated scale. With the tip drawn out so that the rate of delivery is 3 seconds for 0.01 ml. with fully open tap, any degree of control is possible without resort to diminution in hydrostatic pressure.

*The transference with fine delivery tip may be made by directly touching the underneath fluid, and stirring either with fine glass rod—in the Unit technique or otherwise with a bubbling tube (as in Fig. 39). With the 0.5 c.mm. end-point emergence the manipulation error is 0.15 c.mm. (st. dev.) and with the 1.0 c.mm. it is 0.30 c.mm.*

Before the full influence of time on the delivery error was understood, this was obtained as 0.12% in a long series by using the titration method (Chapter XXIV). The delivery here would

be about 2 cm. per second. It is very likely that the delivery error of the horizontal burette obeys the general relation,

$$v = \frac{0.022}{\sqrt{dt}},$$

since the wall fluid goes in the same relation as for the vertical burettes. The particular advantages of the horizontal burette would consist then in avoidance of any drainage error, ease of securing slow and uniform flow without too fine a constriction of tip and previous contact of the emerging fluid with pyrex walls only (described in Part I).

In considering the pipette error here, we have no standard pipette to use in a similar manner to the previous volume levels. Examination of the error of a 0.1 ml. pipette of Ostwald type delivering in 2 seconds before blowing out gave a coefficient of variation of 0.39, but it may be assumed that this error would be considerably less with slow delivery.

**Easily attainable accuracy with the 0.1 ml. technique.** Here we shall take the error of the 0.1 ml. pipette as determined in a given instance, namely 0.39%, the pure delivery error of the burette at about 2 cm. per second as 0.12% and a manipulation error associated with an end-point emergence of 1 small division on the burette giving 0.30 c.mm. (st. dev.). With such data and delivering 0.1 ml. from the burette we obtain a total variable glass error of 0.51% (equation 57).

Where a 0.2 ml. pipette of similar type is used with 0.27% delivery error and titrating 0.2 ml. from the burette, we get a total variable glass error of 0.33%. The total variable error is for these two instances very largely made up of the pipette error. If we used a 1 ml. standard pipette and titrated with 0.2 ml. from the burette the error would be 0.23%.

#### **Approach of the 0.1 ml. to the accuracy of the 25 ml. technique.**

*The 0.1 ml. pipette delivery.* There is every reason to suppose that with suitable reduction in delivery time and fineness of tip, the error here can be made to approach that of the 25 ml. standard pipette, the delivery error proceeding in accordance with the relation already described.

With an internal diameter of 0.09 cm. and delivery length of 16 cm., 96 seconds should be required for the delivery to give an error of 0.03%.

*The burette delivery error (0.1 ml. range).* Seeing that the delivery error was experimentally found as 0.12% with a comparatively fast rate, it may be expected that this will also go in accordance with equation 54, a delivery of 1 cm. in four seconds, giving a presumed error of 0.037%.

*The manipulation error.* Reduction of the hydrostatic pressure for slow delivery with a fine tip touching the fluid surface and stirring by bubbling (Fig. 39) may be expected to reduce the manipulation error to the 0.075 c.mm. (st. dev.) region. A reading limit of 0.2 of a division or 0.2 c.mm. would itself cause an error of about 0.05% (coefficient of variation).

At the same time it will be necessary to deliver 0.15 ml. from the burette to give the required equality with the 25 ml. technique.

The combination of these three errors of pipette (0.030%) and burette (0.037%) deliveries with the manipulation error (0.075 c.mm.), and adding 0.15 ml. from the burette will give a total glass error of 0.069% in accordance with equation 57. This would be practically identical with the error of titrating 25 ml. acid with 25 ml. alkali as above.

**Discriminating and absolute error range with the 0.1 ml. technique.** The discriminating range using duplicate titrations is taken as before at 3 times the total glass error as a coefficient of variation. The difference between this and the absolute error range will depend on the bore uniformity of the burette, assuming the other constant errors suitably allowed for. Here fortunately a high degree of uniformity can be secured by the use of the best thermometer tubing.

The above data for the 0.1 ml. technique are collected in the table on p. 231.

**Note on blood pipetting.** In the approach to the accuracy of the 25 ml. technique one of the main controls lay in diminishing the delivery time. In delivering whole blood, a fine tip is likely to become clogged with inconvenient frequency. Where the procedure allows of increase of fluid volume up to

about five times the blood volume delivered, the easiest way out of such difficulty would consist in using wider tips and carefully washing out. Alternatively some such device as a stopcock could be used to retard flow with conveniently wide tip.

TABLE XXIX

<i>Pipette used.</i>	<i>Variable error of pipette. %</i>	<i>Burette volume titrated ml.</i>	<i>Total variable glass error. %</i>	<i>Discriminating range. %</i>
0.1 (Ostwald type) - -	0.39	0.1	0.51	1.5
0.1 (same) - - -	0.39	0.2	0.43	1.3
0.1 (st. tube, 96 secs. to deliver through 16 cm.) -	0.03 (theor.)	0.15	0.069 (theor.)	0.21 (theor.)
0.2 (Ostwald type) - -	0.27	0.2	0.33	1.0
1.0 (st. tube, 2.5 secs. to deliver 16 cm.) - -	0.13	0.2	0.23	0.7

0.25 ml. *horizontal burette*. Fine grade thermometer tubing. Internal bore 0.09 cm. Manipulation error 0.3 c.mm. or 0.075 c.mm. in the special procedure for the third series in the above Table.

The Ostwald pipettes used delivered with comparative rapidity (about 2 seconds), and are not therefore true to the original Ostwald type.

## THE VARIABLE CHEMICAL ERROR IN TITRATION

If in a series of titrations we were to reduce the original strength of the acid progressively and the alkali correspondingly we would have a growing difficulty in deciding the exact end-point. An error from this source would be introduced, becoming more and more prominent as the original acid strength decreased. This error may be expressed as a standard deviation ( $\sigma_c$ ) and conveniently in millimols, the coefficient of variation being  $\frac{\sigma_c}{q} \times 10^2$ , where  $q$  is the total number of millimols titrated.

The total variable error of the titration ( $v_s$ ) would then be expressible as

$$v_s^2 = v_g^2 + \left( \frac{\sigma_c}{q} \times 100 \right)^2, \quad (58)$$

$v_g$  being the total glass error as previously considered.

**Experimental determination of the variable chemical error.**

The value of  $\sigma_c$  could be obtained experimentally for any type of titration where indicator is added by reducing  $q$  so far, that the glass error is negligibly small, the total error of the titration giving then the chemical error. In such procedure it would be necessary to keep the indicator strength the same throughout, and as low as conveniently possible.

In determining the variable chemical error for acidimetric work, this is largely influenced by the carbon dioxide contained in the water. The carbon dioxide of distilled water, when this is in equilibrium with the carbon dioxide of the air at room temperature, may be calculated to be approximately  $1.7 \times 10^{-5}$  molar from the known solubility of carbon dioxide (1.90 g./litre at 760 mm., ref. 156), and taking the carbon dioxide of the air as 0.04%. Kolthoff (148) has estimated it directly as  $1.5 \times 10^{-5}$ . Distilled water in bulk may, however, contain much greater concentrations of carbon dioxide, arising presumably from gas

burners used in the distilling plant and in bulk only very slowly attains equilibrium with the atmosphere, even with exposed surface. Water from electrically run distilling plants should be practically in equilibrium with the atmosphere, and the use of such water will be considered in the ensuing account.

Also it may be noted that for micro titrations in the central chamber of the Unit, equilibrium with the air may be always assumed owing to the large surface and small depth. In an actual series of such micro titrations of 0.7 ml. of 0.0002*N* acid containing 0.0008% methyl red and 0.0016% methylene blue, with 20% alcohol and using 0.35 ml. barium hydroxide solution from the horizontal burette, a coefficient of variation of 0.78 was obtained, which, allowing for the glass error (found to be 0.20% under the conditions) becomes 0.75% as the variable chemical error (equation 58). This corresponds to  $1.05 \times 10^{-6}$  millimols in the titrating alkali ( $1.4 \times 10^{-4}$  millimols being titrated).

#### Theoretical evaluation of the variable chemical error.

*The end-point error in pH units.* If we suppose titrations carried out with very weak solutions such as 1 ml. of *N*/5000 or of *N*/10,000, and that the exact pH of the end-point reached is subsequently determined, this latter will show a distribution for a series with a certain standard deviation. We should get the same or practically the same distribution if we were titrating a heavily buffered solution around the end-point and adding strong alkali. Such a distribution or standard deviation is immediately related to our visual discrimination, and hence may be expected to vary somewhat from one individual to another. For a single indicator used at or within 0.5 pH from its mid-zone of colour change, the limits of such distribution for careful working is  $\pm 0.1$ —as generally observed (e.g. Bjerrum, 10). This range corresponds also to the differences between buffered indicator tubes in the simplest pH comparators, these differing as a rule by increments of 0.2 pH.

The distribution of the end-point pH will very probably be not quite 'normal', since there must be a central region over which there is the same probability of an observation falling,



but such a deviation from the normal curve will not seriously affect our treatment.

A distribution with practical limits of plus or minus 0.1 *pH* is given by a standard deviation of 0.033 to 0.050 *pH* as we take six or four times its value to cover the range. We may take 0.04 as a probable figure.

If we were to convert the *pH* standard deviation of the end-point into millimols alkali added we could do so in the following equation :

$$\sigma_e = 0.04\beta \times V, \quad (59)$$

where  $\beta$  is  $\frac{dB}{dpH}$ —the buffering power—or the quantity of alkali in millimols necessary to produce unit change of *pH* around the end-point, and may be presumed constant over the small error range in end-point *pH*. This buffering will arise from at least three factors, the hydrogen ion concentration, the carbon dioxide in equilibrium with the air, and the indicator, so that equation 59 may be expanded to

$$\sigma_e = 0.04V(\beta_W + \beta_{CO_2} + \beta_I). \quad (60)$$

We can evaluate each of these buffering factors theoretically.

**The hydrogen ion buffering.** Considering the following two equations,

$$[B'] + [H'] = [OH'] + [A'] \quad (61)$$

$$\text{and} \quad [H'] \times [OH'] = K_W, \quad (62)$$

where equation 61 expresses the necessary relation for electrical neutrality when base is added to a fixed quantity of strong acid and equation 62 the water dissociation, then in differentiating 61 with respect to  $[H']$  and re-arranging, we get

$$\frac{d[B']}{d[H']} = -\frac{[H'] + [OH']}{[H']}. \quad (63)$$

Also, since

$$\begin{aligned} pH &= -\log [H'] \\ &= -0.434 \ln [H'], \end{aligned}$$

then, on differentiating this equation with respect to  $[H']$  and rearranging, we get

$$\frac{d[H']}{dpH} = -2.3[H']. \quad (64)$$

From this and equation (63) we obtain

$$\frac{dB}{dpH} = 2.3\{[H'] + [OH']\} \quad (65)$$

as millimols per ml. (or mols. per litre) and on the acid side of neutrality we may write

$$\frac{dB}{dpH} = \beta_H = 2.3[H']. \quad (66)$$

At a  $pH$  of 5.5 we obtain  $\beta_H$  as  $2.3 \times 10^{-5.5} = 7.3 \times 10^{-6}$ .

**The buffering of the carbon dioxide in equilibrium with the atmosphere.** Considering the equation

$$[H'] \times [HCO'_3] = K[H_2CO_3]. \quad (67)$$

$[H_2CO_3]$  is here a constant when there is equilibrium with the air.

Differentiating with respect to  $[H']$  and re-arranging, we get

$$\frac{d[HCO'_3]}{d[H']} = -\frac{K[H_2CO_3]}{[H']^2} \quad (68)$$

and from equation 66,

$$\frac{d[HCO'_3]}{dpH} = \frac{2.3[H_2CO_3]K}{[H']}. \quad (69)$$

Here, owing to the separate consideration of the hydrogen ion buffering above, the change of  $[HCO'_3]$  may be taken as being exactly equivalent to a corresponding addition of base (it being easily demonstrable that for dilute solutions these various buffer factors can be treated quite independently and additively), so that the required buffering equation is

$$\frac{dB}{dpH} = \beta_{CO_2} = \frac{2.3[H_2CO_3]K}{[H']} \quad (70)$$

in millimols per ml.

Where the  $pH$  is 5.5 and  $[H_2CO_3]$  is  $1.7 \times 10^{-3}$  as given above, we obtain the carbon dioxide buffering for methyl red titrations at 5.5 end-point as

$$\frac{2.3 \times 1.7 \times 10^{-5} \times 10^{-6.4}}{10^{-5.5}} = 4.9 \times 10^{-6} \text{ millimols per ml.}$$

**The indicator buffering.** Considering the equation

$$\begin{aligned} [\text{H}'] \times [\text{I}'] &= [\text{HI}] \times K_I \\ &= (C_I - [\text{I}']) K_I, \end{aligned} \quad (71)$$

where  $C_I$  is the total indicator concentration, and  $[\text{I}']$  the concentration of the ionised substance.

Differentiating with respect to  $[\text{H}']$ , where  $C_I$  is a constant and equating  $d[\text{I}']$  with  $d[B']$  as in previous paragraph:

$$\begin{aligned} \frac{d[\text{B}]}{d[\text{H}']} &= \frac{-C_I K_I}{([\text{H}'] + K_I)^2} \\ \text{and} \quad \frac{dB}{dpH} &= \frac{2 \cdot 3 C_I K_I [\text{H}']}{([\text{H}'] + K_I)^2}. \end{aligned} \quad (72)$$

The  $K_I$  value of methyl red is  $10^{-5.1}$  (157). For methyl red with a molecular weight of 269, an indicator strength of 0.0008% is equivalent to a molar concentration of  $2.98 \times 10^{-5}$ . Inserting these values in equation 72, we get at a pH of 5.5

$$\begin{aligned} \frac{dB}{dpH} &= \frac{2 \cdot 3 \times 2.98 \times 10^{-5.0} \times 10^{-5.1} \times 10^{-5.5}}{(10^{-5.5} + 10^{-5.1})^2} \\ &= 14.0 \times 10^{-6}. \end{aligned}$$

In the example given above for the experimental determination of the variable chemical error, the deliveries of 0.7 ml. of 0.0002*N* acid containing 0.0008% methyl red, etc., were diluted to 1.05 ml. at the end-point, so that the indicator buffering for this dilution would be

$$14.0 \times 0.67 \times 10^{-6} = 9.4 \times 10^{-6} \text{ millimols per ml.}$$

Assembling the values of

$$\beta_H = 7.3 \times 10^{-6}; \quad \beta_{\text{CO}_2} = 4.9 \times 10^{-6} \text{ and } \beta_I = 9.4 \times 10^{-6}$$

in the equation

$$\sigma_e = 0.04 \times V \times (\beta_H + \beta_{\text{CO}_2} + \beta_I),$$

we obtain  $0.9 \times 10^{-6}$  millimols as the theoretical value of the variable chemical error. The experimental value was  $1.05 \times 10^{-6}$ . The agreement here is reasonably good, and an exact correspondence would in fact be only fortuitous.

**Removal or diminution of the variable chemical error.**

In the foregoing account the titrations were taken as conducted

in the presence of atmospheric carbon dioxide. By titrating in a stream of air free from carbon dioxide the effect of this buffer is removed and we can approach nearer to the neutral point. We are still left, however, with some buffering both by hydrogen and hydroxyl ions plus that of the indicator. The latter remains unchanged provided the colour change with  $pH$  can be appreciated with equal delicacy. Under such circumstances we may consider what is the variable chemical error at a  $pH$  of 7.0. We have

$$\sigma_e = 0.04 (\beta_{(H+OH)} + \beta_I) \times V.$$

In general we could titrate micro volumes, using indicator concentrations of about  $1.5 \times 10^{-5}M$  or even less at the end-point, that is, with about half the indicator buffering previously considered, so that

$$\sigma_e = 0.04 (2 \times 10^{-7} + 7 \times 10^{-6}) = 0.3 \times 10^{-6} \text{ millimols/ml.},$$

which represents the minimum variable chemical error or a figure reasonably close thereto.

In such titrations it is assumed that the alkali used is perfectly free from carbon dioxide, the merest trace of which will introduce a comparatively serious error. Such an ideal alkali is realised in practice only when we use barium hydroxide.

**The effect of absorbed ammonia or other weak base on the variable chemical error in acidic titrations.** Where the acid has been used to absorb ammonia, on approaching a  $pH$  of 7.0 we introduce a new error due to the buffering produced by the absorbed ammonia. It is of some interest to inquire into the magnitude of such an error.

The ammonium ion may be conveniently regarded as a weak acid dissociating into ammonia gas and hydrogen ion, with a dissociation constant of  $10^{-9.4}$ . Writing then the general buffering equation for a weak acid as developed above for the indicator buffering (equation 72), we have

$$\frac{dB}{dpH} = \frac{2.3 \times 10^{-9.4} C_{am} \times [H^+]}{(10^{-9.4} + [H^+])^2}$$

and (from 59),

$$\sigma_e = 0.04 \frac{dB}{dpH} \times V = \frac{0.092 \times 10^{-9.4} C_{am} \times [H^+]}{(10^{-9.4} + [H^+])^2} \times V.$$

At  $pH$  values at or below 7.0,  $10^{-9.4}$  in the denominator is negligibly small when compared with  $[H^+]$ , so that

$$\sigma_e = \frac{0.092 \times C_{am} \times 10^{-9.4}}{[H^+]} \times V. \quad (73)$$

The variable error at the end-point is therefore proportional to the amount of absorbed ammonia present. If, however, it is the ammonia and not the acid that is being determined, then the *relative* percentage error will be

$$\frac{0.092 \times 10^{-9.4}}{[H^+]} \times 100 \quad (74)$$

or a figure independent of the ammonia absorbed and dependent only on the  $pH$  and the dissociation constant. Putting in the end-point  $pH$ , we get  $0.11 \times 10^{-2.0}$  or a quite negligible figure at 5.5  $pH$ . At a  $pH$  of 7.0 it becomes 0.04% or something just appreciable.

A similar treatment may be extended to other weak bases.

## CHAPTER XXVIII

### THE RATIONALE OF MICRO TITRATION

THE general variable error of titration is given by the equation already considered (58),

$$v_s^2 = v_e^2 + \left( \frac{\sigma_e}{q} \times 10^3 \right)^2$$

$\sigma_e$ , apart from the indicator, being proportional to the volume at the end-point. For special conditions the form of the equation will alter somewhat, as considered below.

We have seen that for exposed titrations with methyl red  $\sigma_e$  is of the order  $1 \times 10^{-6}$  millimol for each ml. of end-point volume with a possible reduction to  $0.3 \times 10^{-6}$  in a stream of carbon dioxide free air when titrating at a pH of 7.0, though for volumes over a few ml. the titration in a current of carbon dioxide free air, if it is to be conducted efficiently, will be found an inconvenient procedure. These figures apply when the only buffering present at the end-point is the hydrogen and hydroxyl ions of the water, the carbon dioxide of the atmosphere and the indicator. It has also been shown that when ammonia is being determined it begins to introduce a just appreciable buffering of its own at a pH of 7.0, the variable error being then 0.04% and independent of the ammonia concentration.

A certain glass error becomes unavoidable in titration, but where there is no restriction on quantity of material the chemical error above can be reduced to any level considered negligible by increasing  $q$  or the quantity examined. The introduction of an appreciable variable chemical error of the type considered would therefore be merely incorrect technique when we have a large amount of substance. When, on the contrary, there is only a small amount of material for analysis, we may be unable to increase  $q$  sufficiently far to avoid an appreciable chemical

error. In this case we must proceed by diminishing the end-point volume. *This is the chief reason for the employment of a micro volumetric procedure.*

Were it not for this question of variable chemical error, after an initial micro measurement either of volume or quantity we could conduct the remaining analysis by macro volumetric procedure subsequent to dilution.

It will be seen that at the same time any constant deviation of the final  $pH$  from that of the ideal end-point becomes proportionally less as we increase the concentration for titration, or decrease the volume with fixed quantity. This applies to the titration of weak acids and bases as well as to strong, and may be derived formally from the equations in the chapter on the constant chemical error.

The value of a micro technique in this restricted sense has been already pointed out by Rehberg and others (20), but since the titration error referred to is a constant error and may be theoretically determined and so allowed for, it is not so important a reason as that previously given.

With many precipitates, when these are subsequently worked up volumetrically or otherwise, it is necessary also to keep the fluid volume low to avoid appreciable solution.

Besides such reasons of accuracy, we have others also which favour volume reduction with reduced quantities. Many standard solutions, for example, maintain their effective concentrations better in concentrated than in weak solutions, and this applies not only to reagents like permanganate and thio-sulphate, but also to ordinary standard acids and alkalis.

Finally we have reasons of economy, not only of material but often of time, for the carrying out of a micro procedure. Summarising, we may say that the following reasons may determine the choice of a micro volumetric procedure throughout:

- (1) The diminution of the variable chemical error, often the compelling reason for accurate work.
- (2) Diminution of the effect of constant chemical error of the end-point when this deviates from the ideal.
- (3) Diminution of loss by solution with small precipitates intended for quantitative measurement.

(4) The better keeping value of concentrated solutions.

(5) Reasons of economy in material, and often in time.

In the reasons 1 and 2, acidimetric titration is implied, though analogous considerations hold for other volumetric titrations in which there is a change of one spectral colour to another at the end-point. Here as before we will have a variable error of colour discrimination which will be related to concentration of the titrating reagent in excess or defect. An apparent exception is provided in the change from no colour to a coloured end-point. This will be virtually a change from white to some spectral colour, and we can increase the depth of the latter and hence our discrimination by merely increasing the depth of fluid looked through without altering the concentration. The choice of a micro volumetric technique will here depend rather on the other reasons given and the inconvenience of titrating long columns of fluid.

**Experimental exemplification of the interplay of the variable glass and chemical errors.** A large series of titrations—167 in all—were carried out with the same glassware. At the time of these titrations the fuller implications of the error of titration as developed here were not known, but they serve as an illustration of the main reason on which the use of the micro volumetric procedure turns, and of the interplay of the variable glass and chemical errors.

In the series 1 ml. of standard acid was delivered from a Bang burette into a Unit, 0.1 ml. Tashiro's reagent pipetted into the acid and the mixture titrated with standard carbon dioxide free NaOH of the same strength as the acid. Successive series of such titrations were carried out varying the acid and alkali strengths in each series, but keeping the conditions otherwise the same.

These titration series may be examined in relation to the equation (already considered in Chapter XXVII),

$$v_s^2 = v_g^2 + \left( \frac{\sigma_c}{q} \times 10^3 \right)^2,$$

where  $v_s$  is the variable error of the full titration,  $v_g$  that of the glass,  $\sigma_c$  the variable chemical error, and  $q$  the quantity titrated.

Q

C.M.A.



The total glass error can be determined by titrating a series of acid deliveries so strong that the chemical error is negligible, but it may also be determined from its individual components of pipette error (here the delivery error from a Bang burette), burette delivery error and manipulation error. These are related, as already discussed in Chapter XXVI, by the equation:

$$v_g^2 = v_p^2 + v_b^2 + \left( \frac{\sigma_m}{10V} \right)^2.$$

The following values were found experimentally

$$v_p = 0.11\%$$

$$v_b = 0.11\%,$$

$$\sigma_m = 1.3 \text{ c.mm.}$$

$$V_b = 1.0 \text{ ml.}$$

The total variable glass error therefore becomes

$$\begin{aligned} v_g &= \sqrt{0.11^2 + 0.11^2 + 0.13^2} \\ &= 0.203\%. \end{aligned}$$

In a similar way we could build up the variable chemical error from the water, carbon dioxide and indicator buffering and the variable pH error of the end-point as a standard deviation. The total variable chemical error was, however, determined from a titration series so weak that the glass error did not appreciably enter, and was found to be  $1.92 \times 10^{-6}$  millimols.

The result of the series of titrations are then seen in the following table (and in Fig. 46), in which the experimental and theoretical results (from equation 58) are given.

TABLE XXX

Normality of acid and alkali (N).	No. of titrations.	Total variable error of titration ( $v_g$ ).		Titration error expressed as millimols of alkali.	
		Exp.	Theor.	Exp.	Theor.
0.1	52	0.20	0.20	$2.00 \times 10^{-4}$	$2.00 \times 10^{-4}$
0.01	26	0.19	0.20	$1.90 \times 10^{-4}$	$2.00 \times 10^{-5}$
0.001	20	0.30	0.27	$3.00 \times 10^{-5}$	$2.70 \times 10^{-5}$
0.0005	18	0.45	0.42	$2.25 \times 10^{-5}$	$2.10 \times 10^{-5}$
0.0002	51	0.95	0.96	$1.90 \times 10^{-5}$	$1.92 \times 10^{-6}$
0.0001	—	—	1.88	—	$1.88 \times 10^{-6}$

**The question of ideal volumes.** When the need of a micro technique occurs in securing a desired accuracy of determination, the question arises as to how far we are to reduce the volume. Unless we have some guiding principles here to go

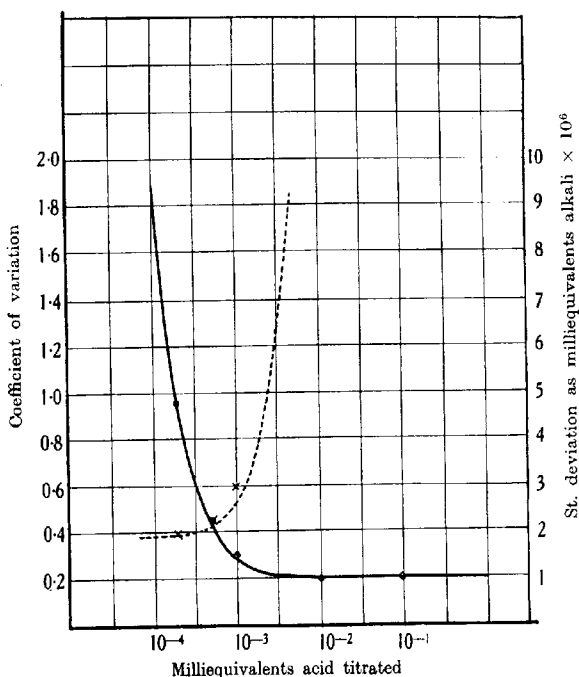


FIG. 46. The curves show the variable error in titrating 1 ml. hydrochloric acid with 1 ml. carbon dioxide free alkali from a 2 ml. Bang burette, using the Tashiro indicator (0.1 ml.). (*Vide text.*) Dots and crosses give the experimental points, the curves being theoretical.

The dotted curve gives the variable error in millimols alkali ( $KOH$ ).

by, the extent of this reduction can only be arbitrary, or determined mostly by the micro apparatus at our disposal.

If we consider the pipettes and burettes in actual use, we shall find that as the glassware is reduced in volume to deliver smaller and smaller amounts, the percentage error of the delivery increases.

As we have already seen, this need not be so, but it is a fact of the conventional usage.

It has been shown in Chapter XXVII that the variable chemical error decreases as the volume decreases, so that at some point we must have an ideal volume when the error is least. We could solve for such a volume if a relation were found connecting the conventional increase of error with decrease of glass volume. This, however, would scarcely be a sound basis, for within limits we can have almost any degree of glass accuracy, and it is preferable to consider firstly what degree of accuracy we want, and then to relate our volumes accordingly, and this we may do approximately as follows.

**A general principle of micro volumetric limitation.** In general it may be said that the end-point volume in micro volumetric procedure should be such that the variable chemical error is at most half the variable glass error that we require. From our glassware we can then secure the accuracy we want in accordance with the principles already outlined. If, for example, we want an accuracy at the 0.1% (coefficient of variation) level, the end-point volume should give a variable chemical error not greater than 0.05%, when the total error of titration with a glass error of 0.1% will not exceed 0.11%. Further volume reduction will secure practically no advantage and only serve to increase the difficulty with which we obtain the necessary glass accuracy.

The question then remains as to how we are to predict the volume that gives us a variable chemical error of, say, 0.05%. Here we may assume as a working relation that the variable chemical error goes proportionally to the end-point volume, though this strictly necessitates the indicator concentration being always the same therein. We may write then

$$\frac{V\sigma_c}{q} \times 10^2 = 0.5v_g, \quad (75)$$

where  $V$  is the end-point volume,  $\sigma_c$  the variable chemical error in millimols per ml. end-point volume,  $q$  the millimols titrated and  $v_g$  the glass error at the level we require.

For acidimetric titration with methyl red  $\sigma_c$  may be taken as approximately  $1 \times 10^{-6}$ , so that in the above example if  $q$  were  $10^{-3}$  and  $v_p = 0.1$ ,  $V$  would require to be 0.5 ml. If our figures were exact it would not be an advantage to have the end-point volume less than 0.5 ml. Since, however, the variable chemical error cannot be accurately predicted for a given set of conditions, it would here be safer to take somewhat less than the volume so derived.

**A special principle of micro volumetric limitation for use with a burette, and fixed pipetted volumes.** When we are restricted to the use of a special burette and a given pipetted volume there is an ideal volume of alkaline solution to add from the burette corresponding to the quantity of acid titrated. The consideration of such ideal volumes is useful only as a working guide and within a certain range of  $q$ . For the methyl red with methylene blue titrations already considered the figure may be arrived at as follows.

We may suppose, for example, that the pipetted acid always contains methyl red in the concentration of 0.0008% with the usual relation of methylene blue, etc. The total error ( $v_t$ ) of the titration may be written

$$v_t^2 = v_p^2 + v_b^2 + \left( \frac{\sigma_m}{10V_b} \right)^2 + \frac{10^4}{q^2} \{ (V_b + V_p) \times w + V_p \times i \}^2. \quad (76)$$

In this equation  $v_p$  and  $v_b$  are the pipetted and buretted delivery errors respectively as coefficients of variation,  $\sigma_m$  is the manipulation error in c.mm.,  $V_b$  and  $V_p$  the buretted and pipetted volumes respectively,  $w$  is the water and carbon dioxide buffering and  $i$  is the indicator buffering, both being multiplied by the standard deviation of the colour change at the end-point expressed as pH units.

On differentiating, equating to zero and re-arranging, we get

$$V_b \left\{ 1 + \frac{V_p}{V_b} \left( 1 + \frac{i}{w} \right) \right\}^{0.25} = 0.032 \sqrt{\frac{q\sigma_m}{w}}. \quad (77)$$

When  $V_b$  is of the same order or greater than  $V_p$  and the indicator strength is maintained as low as conveniently possible, the fourth root of the bracketed expression on

the left will approximate to unity, and we get as a working rule

$$V_b = 0.03 \sqrt{\frac{q\sigma_m}{w}}. \quad (78)$$

Let us suppose, for example, we wished to know the ideal buretted volume in titrating 1 ml. of  $10^{-3} N$  acid, using the methyl red (or methyl red/methylene blue) end-point. At this end-point the water and carbon dioxide (in equilibrium with air) buffering is  $12 \times 10^{-6}$  millimols. If we assume a standard deviation of the colour change at the end-point of 0.04 pH, then  $w$  becomes  $0.5 \times 10^{-6}$ .

We would have then

$$\begin{aligned} V_b &= 42 \sqrt{\sigma_m q} \\ &= 1.4 \sqrt{\sigma_m}, \end{aligned} \quad (79)$$

when

$$q = 10^{-3}.$$

With the Bang 2 ml. burette when  $\sigma_m$  is 1.3 (c.mm.) the value of  $V$  is 1.5 ml. With the horizontal burette when  $\sigma_m$  is 0.30 (minimal emergence of 1 c.mm.), and  $q$  is  $0.2 \times 10^{-3}$  we get 0.24. The value of  $\sigma_m$  or the manipulation error is very largely due to the end-point emergence which gives an error, as already mentioned, expressed by  $\sqrt{\frac{l^2}{12}}$ , where  $l$  is the emergence in c.mm.

It is scarcely necessary to say that these figures are only approximate, but afford at least a rational basis for the order of limitation in micro volumetric titration.

## CHAPTER XXIX

### THE CONSTANT GLASS ERROR

THE constant glass error as here defined is the difference between the mean value of a large number of single deliveries (or fillings) and the nominal value under fixed conditions of temperature, rate of delivery, drainage, etc.

The constant error may always be removed in contrast to the variable error. We can know the probable magnitude of this latter, but under the fixed conditions of our procedure we cannot alter or remove it.

**Determination of the constant glass error.** This determination amounts to the standardisation of the glassware in the usual sense, and may be carried out by determining the mean weight of five deliveries or fillings under the fixed working conditions of the particular glassware.

The standard deviation of the mean value taken from a series of observations is given by

$$\sigma_m = \frac{\sigma}{\sqrt{5-1}} = 0.5\sigma, \quad (80)$$

where five deliveries are taken and  $\sigma$  is the standard deviation of the single delivery from the true mean.  $\sigma_m$  will add to the standard deviation of the single delivery as if it were itself an uncorrelated variable error, so that we have the sum as

$$\begin{aligned} \sigma_s &= \sqrt{\sigma^2 + (0.5\sigma)^2} \\ &= 1.12\sigma. \end{aligned} \quad (81)$$

It will be seen that by taking five deliveries the sum is very little different from the true standard deviation, so that the five delivery mean functions here for all practical purposes as the true mean. Where a volumetric flask is being standardised only one filling need be weighed since the variable error is negligible.

The five deliveries are made into a tared weighing bottle or into a beaker covered with a watch glass, and from the mean weight the volume is determined. In the ensuing section we shall consider how the volume is obtained from the weight at a given temperature and how it may be corrected for a standard temperature.

**Determination and standardisation of the volume from the mean weight at a given temperature.** When the mean weight has been found, the volume at the given temperature or the volume at some standard temperature is then determined from suitable tables (Tables XXXI, XXXII).

We may firstly consider a useful rule for working with factors close to unity. The multiplication or division of such factors can be treated as additions and subtractions.

$$\text{Thus} \quad (1+x)(1+y) = 1+x+y+xy, \quad (82)$$

but if  $x$  and  $y$  are less than one part in 100 or less than 1%, then  $xy$  may be neglected and we have

$$(1+x)(1+y) = 1+x+y. \quad (83)$$

$$\begin{aligned} \text{Similarly} \quad \frac{1+x}{1+y} &= (1+x)(1+y)^{-1} \\ &= (1+x)(1-y+y^2-y^3, \text{ etc.}) \\ &= (1+x)(1-y), \end{aligned}$$

(since  $y^2$ , etc., may be neglected)

$$= 1+x-y, \quad (84)$$

as in the first example.

If we consider the 1 in these factors as understood we can then always work as if they subtracted or added irrespective of their being in fact divisions or multiplications. With the various small corrections to be considered we need only write these down at once with due consideration of their signs, and instead of being parts in 1, we shall consider them to be parts in 100 to avoid undue writing of zeros. Not only can the various temperature and density corrections be treated in this fashion, but also the final allowances for the constant errors in titrations.

If, for example, we have a number of such to deal with and write them as

$$a + b - c + d, \text{ etc.},$$

and require to correct a figure  $X$ , then

$$X \times \left( 1 + \frac{a + b - c + d, \text{ etc.}}{100} \right)$$

gives the required correction.

In the correction of an observed weight of a delivery or a filling of distilled water, we have four possible corrections to consider.

(1) **The density correction.** If the weight of a given volume of water *in vacuo* is  $W$ , then

$$V = \frac{W}{\rho}, \quad (85)$$

where  $V$  is the volume and  $\rho$  is the specific gravity.  $\rho$  may be written as  $\left( 1 - \frac{s}{100} \right)$  and, according to the above treatment, we may write

$$V = W \left( 1 + \frac{s}{100} \right), \quad (86)$$

where  $s$  is 100 times the specific gravity subtracted from unity. The value of  $s$  is given in Table XXXI.

(2) **The air displacement corrections.** The weight in air will be smaller than the weight *in vacuo* by the weight of the air displaced by the water over that of the brass weights. This correction, though small, is larger than may seem likely on a first approach, being approximately 0.1% of the total weight.

If  $V$  is the volume of water, then  $V \left( 1 - \frac{\rho_w}{\rho_b} \right)$  is the volume of air displaced where  $\rho_w$  is the density of the water and  $\rho_b$  the density of the brass weights. This volume of air multiplied by the density of air ( $= \rho_a$ ) at the given temperature and barometric pressure will give the necessary correction.

The full expression for the weight of air displaced in excess of the weights by 100 ml. water is given by

$$a = 100 \left( 1 - \frac{\rho_w}{\rho_b} \right) \times \rho_a \times \frac{273}{T} \times \frac{760}{P}, \quad (87)$$



where  $\rho_a$  is the density of the air at zero centigrade and  $T$  and  $P$  are the temperature (absolute) and barometric pressure.

Here  $\rho_b$  is 8.4 and  $\rho_a$ , 0.00129.

The correction for differences of barometric pressure may be neglected since it will introduce at ordinary levels only a negligible variation.

TABLE XXXI

Temp.	<i>s</i>	<i>a</i>	<i>g</i>	<i>f</i>
10°	0.027	0.109	0.026	0.064
11	0.037	0.109	0.023	0.058
12	0.048	0.109	0.021	0.051
13	0.060	0.108	0.018	0.045
14	0.073	0.108	0.016	0.038
15	0.087	0.107	0.013	0.032
16	0.103	0.107	0.010	0.026
17	0.120	0.107	0.008	0.019
18	0.138	0.106	0.005	0.013
19	0.157	0.106	0.003	0.006
20	0.177	0.105	0.000	0.000
21	0.198	0.105	- 0.003	- 0.006
22	0.220	0.105	- 0.005	- 0.013
23	0.244	0.104	- 0.008	- 0.019
24	0.268	0.104	- 0.010	- 0.026
25	0.293	0.103	- 0.013	- 0.032
26	0.319	0.103	- 0.016	- 0.038
27	0.346	0.103	- 0.018	- 0.045
28	0.374	0.102	- 0.021	- 0.051
29	0.403	0.102	- 0.023	- 0.058
30	0.433	0.101	- 0.026	- 0.064

For the purpose of this small correction also the volume of water may be taken as equivalent to the weight of the water.

The required correction  $a$  is listed in Table XXXI, and is there 100 times the correction for 1 g. water.

Since this correction obviously adds to the weight of water we may write

$$V = W_a \left( 1 + \frac{s+a}{100} \right), \quad (88)$$

where  $W_a$  is now the weight in air, and  $V$  the volume.

(3) The correction for glass expansion. If we have determined the volume of the delivery or filling at a given temperature, when we use the same glassware at another temperature the volume of the glass will have altered, requiring a correction,

therefore, for the glass expansion. This is obviously a correction for use when we are permanently standardising the glass and is quite unnecessary where we want the actual volume after weighing of the immediate delivery or filling. It is convenient to make the correction to some standard temperature and to correct subsequently from this by a table for varying room temperatures.

It is clear that the internal volume of the glassware will alter just to the same extent as if the whole of the internal air space of the empty vessel were solid glass. We must consider therefore the cubical expansion of  $V$  the volume of glass.

The cubical expansion of glass is given as 0.000026 per ml. per degree in the International Critical Tables, so that for  $V$  ml. at  $t^\circ$ , we have the excess volume at  $20^\circ$ :

$$(20 - t) \times 0.000026,$$

and for 100 ml., or practically 100 g. of the weighed water, it will be

$$(20 - t^\circ) \times 0.0026.$$

This percentage correction is listed in Table XXXI as  $g$ , so that

$$V = W_a \left( 1 + \frac{s + a + g}{100} \right), \quad (89)$$

where  $V$  is the volume of a volumetric flask to the mark at  $20^\circ$  when the weight in air of the filling at  $t^\circ$  is  $W_a$ .

The magnitude of this  $g$  correction for a divergence of plus or minus  $10^\circ$  from  $20^\circ$  is 0.026, and could therefore be neglected in practice, but is usually included in a formal treatment.

(4) **The correction for changes in wall fluid with temperature.** This correction does not apply to the volumetric flask in which the fluid volume is not delivered but made up to the mark. The volume of fluid left behind on the walls changes with temperature as determined from the measurement of this volume with the 25 ml. standard pipette. This effect has already been given in Table XXII.

The wall fluid increases as the temperature decreases, and like this will diminish the volume delivered. For every 100 ml. delivered the wall fluid decreases 0.0064 ml. for every degree rise of temperature.\* The correction is listed in Table XXXI as

\* See note at end of chapter.

$f$  and for a range of plus or minus five degrees this correction amounts to 0.036%. We have then for the combination of the four corrections to standardise a pipette or burette

$$V = W_a \left( 1 + \frac{s+a+g+f}{100} \right). \quad (90)$$

Using these four basic corrections, we may consider the various instances in which they arise. These will be considered formally, with subsequent consideration of the corrections we may omit or consider unnecessary to apply in general.

From the principle of treating small errors given at the beginning of the chapter, the required combinations of the corrections can be written down at once as additions or subtractions after a consideration of sign.

#### I. Actual volume of a weighed quantity of water at any room temperature.

Here we have  $s$ , the density correction, and  $a$ , the air displacement correction, to consider. Both will obviously be positive, so that

$$V = W_a \left( 1 + \frac{\alpha}{100} \right), \quad (91)$$

where  $\alpha = s + a$ .  $\alpha$  is given in Table XXXII.

#### II. Standardisation to 20° of a volumetric flask, the weight of the filling being taken at $t^\circ$ .

Here we have to consider  $s$ ,  $a$  and  $g$ , the last being the glass expansion correction. We have to consider only the sign of  $g$ . If it is less than 20° the effect of  $g$  must be positive since the volume will expand on heating to 20°. Making  $g$  always positive in the equation will mean positive values below 20° in table and negative values above, as already considered. We have then

$$V = W_a \left( 1 + \frac{\beta}{100} \right), \quad (92)$$

where  $\beta$  is  $(s + a + g)$  and is listed in Table XXXII.

#### III. Standardisation of pipette or burette mean delivery to a standard temperature of 20°; the mean weight of five deliveries being taken at $t^\circ$ .

Here we have  $s$ ,  $a$ ,  $g$  and  $f$  to consider. We have then

$$V = W_a \left( 1 + \frac{\gamma}{100} \right), \quad (93)$$

where  $\gamma$  is  $(s + a + g + f)$ .

The value of  $\gamma$  is listed in Table XXXII.

TABLE XXXII

Temp.	$\alpha$	$\beta$	$\gamma$	$\delta$	$\epsilon$	$\zeta$	$\eta$	$\theta$
10°	0.136	0.162	0.226	-0.026	-0.090	-0.124	-0.060	0.150
11	0.146	0.169	0.227	-0.023	-0.081	-0.117	-0.059	0.140
12	0.157	0.178	0.229	-0.021	-0.072	-0.108	-0.057	0.129
13	0.168	0.186	0.231	-0.018	-0.063	-0.099	-0.054	0.117
14	0.181	0.197	0.235	-0.016	-0.054	-0.088	-0.050	0.104
15	0.194	0.207	0.239	-0.013	-0.045	-0.077	-0.045	0.090
16	0.210	0.220	0.246	-0.010	-0.036	-0.066	-0.038	0.074
17	0.227	0.235	0.254	-0.008	-0.027	-0.049	-0.030	0.057
18	0.244	0.249	0.262	-0.005	-0.018	-0.034	-0.021	0.039
19	0.263	0.265	0.271	-0.003	-0.009	-0.017	-0.011	0.020
20	0.282	0.282	0.282	0.000	0.000	0.000	0.000	0.000
21	0.303	0.300	0.294	0.003	0.009	0.018	0.012	-0.021
22	0.325	0.320	0.307	0.005	0.018	0.038	0.025	-0.043
23	0.348	0.340	0.321	0.008	0.027	0.059	0.040	-0.067
24	0.372	0.362	0.336	0.010	0.036	0.081	0.075	-0.091
25	0.396	0.383	0.351	0.013	0.045	0.103	0.071	-0.116
26	0.422	0.406	0.368	0.016	0.054	0.126	0.088	-0.142
27	0.449	0.431	0.386	0.018	0.063	0.151	0.106	-0.169
28	0.476	0.455	0.404	0.021	0.072	0.176	0.125	-0.197
29	0.505	0.482	0.424	0.023	0.081	0.203	0.145	-0.226
30	0.534	0.508	0.444	0.026	0.090	0.230	0.166	-0.256

IV. A volumetric flask standardised for 20° is used at some other temperature,  $t^\circ$ , the volume correction being required.

The sum of the corrections for IV and II must give that for I, since if a pipette is standardised to 20° at a room temperature of  $t^\circ$  and then wanted for immediate use, obviously on correcting back to  $t^\circ$ , we would obtain the actual volume of the delivery examined, or the value considered in I, consequently the required correction is

$$V_t = V_{20} \left( 1 + \frac{\delta}{100} \right), \quad (94)$$

where  $\delta$  is  $(s + a) - (s + a + g) = -g$ .

$\delta$  is listed in Table XXXII and is merely minus the  $g$  value of the previous table.

V. A pipette or burette standardised for  $20^\circ$  is used at some other temperature,  $t^\circ$ , the extra volume correction for delivery being required.

Here as with IV the sum of the corrections in II and V must give that in I, so that we may write

$$V_t = V_{20} \left( 1 + \frac{\epsilon}{100} \right), \quad (95)$$

where  $\epsilon$  is  $(s+a) - (s+a+g+f)$  or  $-(f+g)$ .

This correction is listed in Table XXXII.

**Reduction in the number of the volume corrections.** For work short of the finest all the above corrections may be reduced to one, namely,  $\alpha$ , i.e. for  $\beta$  and  $\gamma$  we may write  $\alpha$ , and consider  $\delta$  and  $\epsilon$  as negligible.  $\alpha$  is the percentage factor for converting a weighed quantity of water to a volume.

**Example of the corrections and factors in use.**

Mean weight of five deliveries from a 25 ml. pipette

24.9275 g.

Temperature of water  $23.5^\circ$ .

It is required to standardise the pipette for  $20^\circ$ .

The value of  $\frac{(24.9275 - 25.0000)}{25} \times 100 = -0.290$ ,

so that  $W = 25 \left( 1 + \frac{(-0.290)}{100} \right)$ .

From I above the  $\gamma$  correction is required, so that

$$\begin{aligned} V &= 25 \left( 1 + \frac{\gamma - 0.290}{100} \right) \\ &= 25 \left( 1 + \frac{0.038}{100} \right), \end{aligned}$$

$\gamma$  from Table XXXII being  $0.328$  at  $23.5^\circ$ .

The factor of the pipette for  $20^\circ$  is therefore most conveniently expressed as  $P_{20} = +0.038$ .

This factor may then be kept as a constant standardising factor of the 25 ml. pipette at  $20^\circ$ .

The actual volume of the delivery at 20° from the above is

$$25 \left( 1 + \frac{0.038}{100} \right) \quad \text{or} \quad 25.008 \text{ ml.}$$

**2nd example.** A 25 ml. pipette has a factor at 20° ( $P_{20}$  as in above example) of +0.084, and is used at a room and water temperature of 25°. What is the factor for this temperature?

We have from IV above

$$\begin{aligned} V &= V_{20} \left( 1 + \frac{\delta}{100} \right) \\ &= 25 \left( 1 + \frac{P_{20}}{100} \right) \left( 1 + \frac{\delta}{100} \right) \\ &= 25 \left( 1 + \frac{P_{20} + \delta}{100} \right). \end{aligned}$$

$P_{20}$  is +0.084 and  $\delta$  from Table XXXII is 0.013, so that the factor for 25 degrees is

$$0.084 + 0.013 = 0.097.$$

Thus the final factor is got at once by reading  $\delta$  from Table XXXII and adding to  $P_{20}$ .

From the above two examples it will be seen that all the glassware should be standardised for 20° with their respective percentage factors noted. From these any other correction is very easily obtained simply by adding or subtracting the figure obtained from the table.

The other factors of Table XXXII are explained in the ensuing chapter concerning the constant chemical correction.

**The corrections with analytical solutions.** The corrections considered above may be taken to apply when using all volumetric solutions up to 0.1*N* concentrations, and even with solutions of normal concentration, with some few exceptions.

With regard to these exceptions, the volumes delivered from a 100 ml. pipette of a number of ordinary reagents up to normal strength have been investigated by Schloesser (157), and with the exception of normal sodium carbonate, Fehling's solution II, ferric chloride (1 ml.=0.012 g. Fe) and 0.1*N* iodine, the volume was inappreciably different from a similar

delivery of water. Even these exceptions differed only by the following amounts:  $-0.035\%$ ,  $-0.198\%$ ,  $-0.035\%$  and  $+0.025\%$  respectively.

Concerning the density changes of such solutions with temperature, these practically agree with water up to  $0.1N$  strength, and again even at  $1.0N$  strength differ but little. An exception to this is  $1.0N$  sodium hydroxide, the density change of which is about  $50\%$  greater than that of water around  $20^\circ$ .

*Note on the temperature correction for wall fluid ( $f$ , Table XXXI).*

Since the temperature effect is probably proportional to the volume of wall fluid, this varying in accordance with the factors described in Chapter XXIV, it follows that the  $f$  correction listed is valid only for standard glassware within a certain volume range. For 20–50 ml. deliveries however it may be taken as giving an accuracy to  $0.01\%$  over  $\pm 5$  degrees C. Further adjustment of the correction to varying wall fluid may be made from the relation this bears to the variable error. It may be assumed in short that the factor  $f$  will vary as  $x/0.025$  where  $x$  is the variable error and  $0.025$  that of the standard 25 ml. pipette. Factors  $\gamma$ ,  $\epsilon$  and  $\eta$  in Table XXXII will also be affected since they contain the correction  $f$ .

## CHAPTER XXX

### THE CONSTANT CHEMICAL ERROR

THE constant chemical error in titration may be considered under three headings :

- (1) Constant error of impurity in the standardising substance.
- (2) Constant error in the standard solutions under the given conditions, the actual strength differing from the nominal.
- (3) Constant error resulting from deviation of the mean end-point from the ideal end-point.

**(1) Constant impurity in the standardising substance.**

The standardising substances such as sodium oxalate, sodium carbonate, potassium biphthalate, benzoic acid, etc., are now prepared commercially to a degree of purity of about 99.9%, referring to the dried substance.

For acidimetric standardisation of standard acidic solutions sodium oxalate (equivalent weight 66.98) is probably the best, and has been strongly recommended by Sørensen (158), by Kolthoff (148) and others.

It has the advantage over sodium bicarbonate (which is converted to carbonate on heating and subsequently weighed as such) in that an excess loss of carbon dioxide with conversion to hydroxide on heating is of no consequence, the substance being accurately weighed for use before it is decomposed.

Sodium oxalate can be prepared to an accuracy of at least 99.96% purity without much difficulty.

It may also be mentioned that the preparation of standard hydrochloric acid to a possible accuracy of 99.99% is claimed for the Hulett and Bonner (159) constant boiling mixture methods.

**Expression of the constant error in the standardising substance.** This may be expressed in a similar way to the constant errors of the glassware, thus :

$$M = M_0 \left( 1 + \frac{i + a_m}{100} \right). \quad (96)$$



Here  $M$  is the actual weight of the perfectly pure substance,  $M_0$  is the nominal weight in air,  $i$  and  $a_m$  are the impurity and air displacement errors as percentages. In the particular instance of sodium oxalate as prepared above, these two are opposite in sign and practically identical in amount, so that the whole correction for impurity and air displacement could be neglected.

(2) **Constant error in the standard solutions.**

(a) *An equivalent weight of a solid or some fraction thereof is weighed and dissolved in water to the mark in a volumetric flask at  $t^\circ$ ; it is required to standardise the solution for  $20^\circ$ .*

The flask may be at first supposed to have exactly the nominal volume at  $t^\circ$ . After making up the solution to the mark, if the flask and contents are changed from  $t^\circ$  to  $20^\circ$  the meniscus will rise or fall above or below the mark, the glass expansion itself having no effect on the alteration of strength produced. This is referable only to the density change.

If the temperature is below  $20^\circ$ , then on changing to  $20^\circ$  the volume increases and the solution strength decreases, so that the ultimate sign of the correction must then be minus, and we may write  $(s_t - s_{20})$  as the factor, since this from Table XXXI gives a minus sign.

In the above we proceeded as if the flask had no factor at  $t^\circ$ . Assuming its factor at  $20^\circ$  to be  $F_{20}$ , then its factor at  $t^\circ$  ( $F_t$ ) is given by  $F_{20} - g$ , where  $g$  is the glass expansion factor in Table XXXI. If the sign of this factor is plus its effect on the strength of the solution must be minus, so that the whole factor is

$$\begin{aligned} S_{20} &= (s_t - s_{20}) - (F_{20} - g) \\ &= s_t - s_{20} + g - F_{20} \\ &= \zeta - F_{20}, \end{aligned} \tag{97}$$

where  $\zeta$  is  $(s_t - s_{20} + g)$  and is listed in Table XXXII.

(b) *An exactly weighed standardising solid is dissolved and titrated from a burette at  $t^\circ$ . It is required to standardise the burette solution for  $20^\circ$ .*

The solution is standardised firstly as follows :

If  $X$  is the expected number of ml. and  $(X+x)$  the actual, then we may write

$$\frac{(X+x) - X}{X} \times 100 = D. \quad (98)$$

$D$  will be positive if  $x$  is positive, but the strength of the solution must then decrease from the nominal, so that  $D$  will require a minus sign.

For standardisation we must now combine the factor for the density change and the factor of the burette.

The density and glass change will enter in the same way as in the previous example, but the effect of temperature on the wall fluid must be also considered, so that

$$S_{20} = \zeta + f - B_{20} - D,$$

(where  $B_{20}$  is the burette percentage factor)

$$= \eta - B_{20} - D. \quad (99)$$

$\eta$  is listed in Table XXXII and is given by  $(s_t - s_{20} + g + f)$  from Table XXXI.\*

(c) *A pipette delivery of a fluid standardised for 20° is titrated from a burette at t°. It is required to standardise the burette fluid for 20°.*

Here the effect of the density change and of wall fluid will be the same for both fluids and may be omitted. We can regard the whole titration as taking place at 20°.

The required standardisation factor is here

$$S_{20} = P_{20} - B_{20} - D, \quad (100)$$

where  $P_{20}$  and  $B_{20}$  are the pipette and burette factors, and  $D$  the value from equation 98, being the immediate titration factor.

It is obvious that the pipette and burette factors must go in opposite ways. If the pipetted fluid has already a factor at 20 ( $S'_{20}$ ), this is included as

$$S_{20} = P_{20} - B_{20} + S'_{20} - D. \quad (101)$$

The above three are standardisation corrections for solutions.

\* See note at end of previous chapter.

If the solutions are standardised for  $20^\circ$ , then on titrating at other temperatures no further temperature corrections need be considered, since glass and solutions in the different glass-ware will be equally affected. It may be noted, however, that wherever we require to titrate a measured volume against a weighed quantity, we then need to correct for altered temperature.

(d) *A solution in bottle has a factor for  $20^\circ$  ( $=S_{20}$ ); its factor at  $t^\circ$  is required.*

Here as special factor we require to consider only the density change. The colder the solution the more it increases in strength, and since  $s_{20} - s_t$  is positive for  $t$  values below  $20$ , the required factor is obtained from

$$\begin{aligned} S_t &= S + (s_{20} - s_t) \\ &= S + \theta. \end{aligned} \quad (102)$$

The value of  $(s_{20} - s_t) = \theta$  is listed in Table XXXII.

(e) *A burette or pipette at  $t^\circ$  delivers a certain volume of a standard solution; it is required to find the exact volume of the nominal strength ( $N/10$ ,  $N/20$  or other such) delivered.*

The special factor entering here will be necessarily made up of  $s_{20} - s_t$  and  $g + f$ ; we have also the burette and solution factors for  $20^\circ$  to consider in the full equation.

In a similar way to the foregoing sections, if  $V_t$  is the required volume correction, then

$$\begin{aligned} V_t &= S_{20} + B_{20} + (s_{20} - s_t) - (g + f) \\ &= S_{20} + B_{20} - \eta. \end{aligned} \quad (103)$$

( $\eta$  is already listed in Table XXXII.)

**Some examples of the use of foregoing constant chemical corrections.**

EXAMPLE 1. 25 ml. of a 0.1N hydrochloric acid are pipetted into a flask and titrated with 0.1N alkali from a burette. It is required to standardise the alkali against the acid. Room temperature  $16^\circ$ .

$P_{20}$ percentage factor of pipette at $20^\circ$	$= +0.140,$
$B_{20}$ percentage factor of burette at $20^\circ$	$= -0.067,$
$S_{20}$ percentage factor for solution strength of acid at $20^\circ$	$= +0.348.$

From (c) above (equation 101) the percentage correction is given by

$$S_{16} = P_{16} - B_{16} + S_{16}' - D.$$

$D$  is given (see equation 98 above) by

$$\frac{25.15 - 25.0}{25} \times 100 = +0.600.$$

We have then  $S_{20} = +0.140 + 0.167 + 0.348 - 0.600$   
 $= +0.055,$

which is the percentage factor for the alkali at  $20^\circ$ .

The strength of the 0.1N alkali at 20 is therefore

$$\frac{N}{10} \times \left(1 + \frac{0.055}{100}\right) = \frac{N}{10} (1.00055).$$

EXAMPLE 2. A 10 ml. pipette is used to deliver 0.1N sulphuric acid at  $26.0^\circ$ . What is the exact equivalent of the acid delivered?

$P_{10}$  is  $+0.134$ .

$S_{20}$  is  $-0.467$ .

From section (e) (equation 103),  $V_t = P_{20} + S_{20} - \eta$ , and from Table XXXII  $\eta$  is 0.088, so that the percentage factor is

$$+0.134 - 0.467 + 0.088 = -0.245.$$

$10 \times 0.1 \times \left(1 - \frac{0.245}{100}\right)$  milliequivalents are therefore delivered  
 $= 0.9975.$

**Résumé of constant corrections for glassware and standard solutions.** The various special factors, etc., are conveniently grouped in the following table :

TABLE XXXIII

Requirement.	Special factor (%).	Full factor (%).
1. Actual volume of weighed water.	$\alpha = (s + a).$	$\alpha$
Glass standardisation to $20^\circ$ .		
2. Volumetric flask at $t^\circ$ .	$\beta = (s + a + g).$	$F_{20} = \beta + D$ , where $D = \frac{W - V_N}{W} \times 100.$ $V_N$ is the nominal volume; $W$ the weight in air.
3. Pipette or burette at $t^\circ$ .	$\gamma = (s + a + g + f^*).$	$P_{20}$ (or $B_{20}$ ) $= \gamma + D$ . $(D$ as in previous.)
Volume of glass filling or delivery at $t^\circ$ from standard value at $20^\circ$ .		
4. Volumetric flask (standardised at $20^\circ$ ).	$\delta = (-g).$	$F_t = F_{20} + \delta.$
5. Pipette (or burette).	$\epsilon = (-f - g).$	$P_t = P_{20} + \epsilon.$

\* See note at end of previous chapter.

(Continued on p. 262)

TABLE XXXIII—Continued

Requirement.	Special factor (%).	(Full factor %).
<i>Solution standardisation to 20°.</i>		
6. Solid dissolved to mark in flask at $t^\circ$ .	$\zeta = (-s_{20} + s_t + g)$ .	$S_{20} = \zeta - F_{20}$ .
7. Burette solution $v$ solid at $t^\circ$ .	$\eta = (-s_{20} + s_t + g + f)$ .	$S_{20} = \eta - B_{20} - D$ , $D = \frac{T - T_N}{T} \times 100$ , where $T$ is the actual titration figure and $T_N$ the nominally expected figure.
8. Burette solution $v$ pipetted solution.	None.	$S_{20} = P_{20} - B_{20} + S_{20}' - D$ (symbols as in previous). $S_{20}'$ = strength of pipetted solution.
<i>Correction for molarity or equivalents at <math>t^\circ</math> from value at 20°.</i>		
9. Solution strength at $t^\circ$ .	$\theta = (s_{20} - s_t)$ .	$s_t = S_{20} + \theta$ .
10. Equivalents delivered at $t^\circ$ by pipette or burette.	$-\eta = (s_{20} - s_t - g - f)$ .	$V_T = -\eta + P_{20} + S_{20}$ .

**Reduction of number of factors.** If  $g$  and  $f$  be considered negligibly small, all the special factors above reduce to two,  $\alpha$  and  $\zeta$ . For  $\alpha, \beta, \gamma$  we have simply  $\alpha, \delta$  and  $\epsilon$  disappearing while  $\zeta, \eta, \theta$  become  $\zeta, \zeta$  and  $-\zeta$ . A study of Table XXXII will help to decide what factors may be neglected in special circumstances.

(3) **Constant error arising from deviation of end-point pH from the ideal figure.** In titrating strong acid with strong base in pure dilute solutions from

$$[B'] + [H'] = [OH'] + [A'], \quad (104)$$

which is a necessary relationship for electric neutrality, we have  $[H']$  equal to  $[OH']$  when  $[B']$  is equal to  $[A']$ , or where there is present an exact equivalence in the titration, the ideal end-point being therefore the theoretically neutral point, which we may take here as having a pH of exactly 7.0.

Where there is a divergence from this equivalence, we have

$$\frac{[B'] - [A']}{[A']} \times 100 = \frac{[OH'] - [H']}{[A']} \times 100. \quad (105)$$

The expression on the right may be written

$$\frac{-[H']}{[A']} \times 100, \quad (106)$$

when the end-point is one or more pH units from 7.0, and gives the percentage error. Here

$$[A'] = C_A \times \frac{V_0}{V};$$

$C_A$  being the original acid concentration and  $V_0$  and  $V$  the original and end-point volumes. It is of some interest to assess this error for the methyl red indicator with varying strengths of acid for titration. We shall suppose that the acid is titrated with an equal volume of alkali, so that the error becomes

$$\frac{-10^{-5.5}}{0.5C_A} \times 100 = \frac{-2 \times 10^{-3.5}}{C_A}, \quad (107)$$

where  $C_A$  is the original concentration of the acid.

The following short table gives the values of the error for different acidic concentrations (the error being independent of actual amounts).

TABLE XXXIV

<i>Acid concentration.</i>	<i>% error with equal volume of alkali.</i>	<i>% error titrating with alkali volume one-fifth the acid volume.</i>
0.1N	0.0063	0.0038
0.01N	0.063	0.038
0.001N	0.63	0.38
0.0005N	1.26	0.76
0.0002N	3.15	1.90
0.0001N	6.30	3.80

When titrating 0.0002N acid in the Unit therefore (as for the blood ammonia) we have a constant error in the alkali used of 1.9%. This does not enter into the ammonia determination,

since it is the difference between two titrations brought to the same end-point that is used to measure the ammonia. It does enter, however, into the standardisation of the alkali with the acid, 1.9 per cent. more alkali being required for exact equivalence.

When titrating with methyl red using equal alkaline volume, the constant error reaches 0.01% when the original acid strength is 0.063*N* or in the region of *N*/20. The variable error when the end-point mixture is in equilibrium with the atmospheric carbon dioxide is (from Chap. XXVII) approximately

$$\frac{1 \times 10^{-6} \times 2V}{V \times C_A} \times 100 = \frac{10^{-4} \times 2}{C_A} = 0.003\%,$$

with a range of about 0.01% or the same order as the constant error. This variable error maintains the same relation to the constant error throughout, but whereas the constant error may be always allowed for, the variable error cannot.

**Constant error in the titration of a weak acid or alkali.** The *pH* of exact equivalence when titrating a weak acid is not 7.0, but approaches this as the concentration for titration diminishes. For dilute solutions the theory of this equivalence and associated errors may be treated as follows.

With a weak acid, the equation

$$[\text{HA}] + [\text{A}'] = [\text{B}'] \quad (108)$$

must hold at the equivalent point—the amount of strong base added being equal to the total weak acid, dissociated and undissociated.

Also the total negative ions must be equal to the total positive,

$$[\text{A}'] + [\text{OH}'] = [\text{B}'] + [\text{H}']. \quad (109)$$

From these two equations, we have

$$\begin{aligned} [\text{H}'] &= [\text{OH}'] - [\text{HA}] \\ &= \frac{K_w}{[\text{H}']} - [\text{HA}], \end{aligned} \quad (110)$$

where  $[\text{H}']$  is the hydrogen ion concentration of the ideal end-point.

$[\text{HA}]$  may now be expressed in terms of  $[\text{H}^+]$  and the total weak acid concentration  $C_A = [\text{A}'] + [\text{HA}]$ ,

for  $[\text{H}^+] \times [\text{A}'] = K_a \times [\text{HA}]$ ,  
so that

$$[\text{H}^+](C_A - [\text{HA}]) = K_a[\text{HA}]. \quad (111)$$

From equations 111 and 110 we get

$$[\text{H}^+]^3 + [\text{H}^+](K_a + C_a) - K_w[\text{H}^+] - K_wK_a = 0, \quad (112)$$

where  $K_a$  is greater than  $10^{-7}$  this reduces to

$$[\text{H}^+] = \sqrt{\frac{K_wK_a}{C_a + K_a}}, \quad (113)$$

$[\text{H}^+]$  being the hydrogen ion of the ideal end-point. The  $p\text{H}$  value is given by

$$p\text{H} = 0.5 \log \left( 1 + \frac{C_a}{K_a} \right) + 7.0. \quad (114)$$

Similarly for the titration of a weak alkali,  $K_b$  greater than  $10^{-7.0}$ ,

$$[\text{OH}'] = \sqrt{\frac{K_wK_b}{C_b + K_b}}$$

or

$$[\text{H}^+] = \sqrt{\frac{K_w(C_b + K_b)}{K_b}}, \quad (115)$$

the ideal  $p\text{H}$  is given for the weak alkali by

$$p\text{H} = -0.5 \log \left( 1 + \frac{C_b}{K_b} \right) + 7.0. \quad (116)$$

**Constant error resulting from deviations from the ideal end-point in titrating weak acid or weak base.** For acids or alkalis with dissociation constant of the order of  $10^{-7}$  or greater, we can devise useful approximate expressions. If we take the total buffering of a weak acid in water it may be written (according to Chapter XXVII) as

$$\frac{dB}{dp\text{H}} = \frac{2.3C_aK_a[\text{H}^+]}{\{[\text{H}^+] + K_a\}^2} + 2.3\{[\text{H}^+] + [\text{OH}']\}, \quad (117)$$

whence considering  $dp\text{H} = \frac{-d[\text{H}^+]}{2.3[\text{H}^+]}$ ,

$$\text{we obtain } \frac{dB}{d[\text{H}^+]} = \frac{-C_aK_a}{\{[\text{H}^+] + K_a\}^2} - \left\{ \frac{[\text{H}^+] + [\text{OH}']}{[\text{H}^+]} \right\}. \quad (118)$$



Since at the ideal end-point for a weak acid  $[\text{OH}']$  will be much greater than  $[\text{H}']$ , the latter may be neglected in the second expression on right. Then on integrating, we get

$$\begin{aligned} B_1 - B_2 &= \frac{C_a K_a}{[\text{H}']_1 + K_a} - \frac{C_a K_a}{[\text{H}']_2 + K_a} + [\text{OH}']_1 - [\text{OH}']_2 \\ &= C_a K_a \left\{ \frac{[\text{H}']_2 - [\text{H}']_1}{([\text{H}']_1 + K_a)([\text{H}']_2 + K_a)} \right\} + [\text{OH}']_1 - [\text{OH}']_2. \quad (119) \end{aligned}$$

In this the value of  $[\text{H}']_1$  and  $[\text{H}']_2$  may be taken as negligibly small compared with  $K$ , so that

$$B_1 - B_2 = \frac{C_a}{K_a} \{ [\text{H}']_2 - [\text{H}']_1 \} + [\text{OH}']_1 - [\text{OH}']_2. \quad (120)$$

Here  $C_a$  must be read as the total weak acid concentration in the end point volume.

The base required per unit volume to produce a change in the actual  $[\text{H}']$  of the end point to the ideal value given above may then be calculated from this equation, and expressed as a percentage of the total base added or the total acidic equivalent.

In this treatment the carbon dioxide of the air has been ignored, which can only be done when for weak concentrations we titrate in a current of carbon dioxide free air. Otherwise we should have extreme errors in titrating a weak acid to an alkaline end-point.

## CHAPTER XXXI

### VOLUMETRIC ERROR IN KJELDAHL NITROGEN ANALYSES

An analysis of the volumetric error in the classical macro-Kjeldahl determination and a comparison with that using the micro-diffusion principle. This is taken as providing a practical example for the foregoing system of error analysis. It will be considered firstly in relation to the error between two successive determinations carried out in duplicate, using the same glassware and the same solutions and quantities of material. This will give a measure of the discriminating delicacy of the procedure. Secondly, the absolute error will be considered, involving the introduction or allowance for the constant glass and chemical errors.

Since it will make it somewhat more general to consider the estimation carried out on a fluid which is delivered from a pipette, this procedure will be considered. The following example then may be cited.

3 ml. urine pipetted into incinerating flask.

20 ml. 0.1N HCl pipetted into receiving flask.

40 ml. 0.05N NaOH required to titrate a trial delivery of acid.

10 ml. 0.05N NaOH required to titrate back after distilling.

*Standardisation of alkali.* 45.0 ml. used in standardisation against potassium biphthalate or benzoic acid (or alternatively standardisation of acid against oxalate, with subsequent standardisation of alkali against acid.

**1. The discriminating range.** By discriminating range is meant here the percentage range of error that could occur between two determinations carried out on the same solution and with the same glass and standard solutions. In Chapter XXVI this range was considered for duplicate titrations being independent of the constant errors, including the bore variation of the burette used.

The discriminating range when dealing with duplicates is the same as that arising for the variable error of a single determination from the mean of a large number (Chapter XXVI). Here there enters practically no variable chemical error owing to the strength of the solutions used, so that it may be considered entirely as a glass and glass manipulation error. We have then the following constituent errors to consider:

	<i>Variable error, coeff. of variation.</i>	<i>Variable error as millimols (NaOH).</i>
3 ml. pipetted	0.072	$30 \times 0.05 \times 7.2 \times 10^{-4} = 10.8 \times 10^{-4}$
20 ml. acid delivered	0.028	$40 \times 0.05 \times 2.8 \times 10^{-4} = 5.6 \times 10^{-4}$
10 ml. back titration	0.152	$10 \times 0.05 \times 15.2 \times 10^{-4} = 7.6 \times 10^{-4}$

Summation of the above variable errors gives as millimols

$$\sigma^2 = 10^{-8}(10.8^2 + 5.6^2 + 7.6^2)$$

$$= 10^{-8}(205)$$

and

$$\sigma = 14.3 \times 10^{-4}.$$

The total millimols of  $N$  (as  $NH_3$ ) are  $(40 - 10) \times 0.05 = 1.5$ . The variable error of the determination as a coefficient of variation is therefore

$$\frac{14.3 \times 10^{-4}}{1.5} \times 100 = 0.095\%.$$

The standard deviation from the mean of a large number of similar determinations is hence  $0.095\%$  with a range of  $\pm 0.28\%$ . If a 5 ml. standard pipette were used instead of the 3 ml. it would be  $\pm 0.24$ . This range is also the discriminating range if duplicates are carried out with each solution.

**Absolute range of error.** In the discriminating error range, the variable error of titration of a delivery of unchanged acid by the alkali did not enter, since the same titration would suffice for the two analyses to be discriminated.

Concerning the absolute error of the single analysis, however, we require to know the exact value in equivalents of the acid delivered and its corresponding alkali value, and in

determining this, further variable errors are necessarily introduced.

We could suppose the acid standardised by titration of a weighed solid and the alkali in turn by titration of the preliminary acid delivery. It will mean then the summation of the variable error of standardising a weighed solid, and the variable error of titrating a pipetted volume, a sum slightly (but scarcely significantly) larger than where the alkali is also standardised against a solid.

We have then

	<i>Variable error (from considerations in Chapter XXVI).</i>	<i>Variable error in millimols (NaOH).</i>
Preliminary titrations of 20 ml. of 0.1N acid, using 40 ml. alkali -	0.052	$10.4 \times 10^{-4}$

This added to the previous variable discriminating error of  $14.3 \times 10^{-4}$ , gives

$$\begin{aligned}\sigma &= 10^{-4} \sqrt{10.4^2 + 14.3^2} \\ &= 17.7 \times 10^{-4}.\end{aligned}$$

This gives a coefficient of variation of

$$\frac{17.7 \times 10^{-4}}{(40 - 10) \times 0.2} = 0.118\%.$$

To this now adds the coefficient of variation due to the acid standardisation, and we may assume this is carried out with a 40 ml. delivery from a 50 ml. burette in the usual way, and giving a coefficient of variation of 0.044, so that the total becomes

$$\begin{aligned}v_t &= \sqrt{0.118^2 + 0.044^2} \\ &= 0.126,\end{aligned}$$

giving a range of 0.38%.

(The variable error of acid standardisation adds in this way, because it will affect each member of the previous error—expressed as millimols—in a proportionate manner, but will at

the same time be uncorrelated with or independent of the total previous error, *i.e.* of the 0.118%.)

**Bore tolerance of burette.** In the above it has been taken that the bore variation of the burette came under the heading of constant error, but the bore variation may be inconvenient to determine, though given in the certificate of standardisation supplied with the best class burettes. If we go instead by the full 'tolerance' of the burette, this can be included in a consideration of the limits of the variable error. In the above example, the difference between the 40 ml. of the preliminary titration and the 10 ml. of the back titration may be up to this full tolerance limit or 40-60 c.mm. with different kinds of first-class burette; 40 c.mm. tolerance will be taken here, since such burettes may be readily procured.

At the same time an allowance for bore or (as here) full tolerance comes into the standardisation, but will act in the opposite direction. If we take it that the 40 ml. on the burette is really 40 + 0.040 and the 10 ml. exact, then the alkali standardisation correction is

$$\frac{-0.040}{40} \times 100 = -0.100\%$$

whereas the correction for the ammonia absorbed would amount to

$$\frac{+0.040}{(40.04 - 10)} \times 100 = +0.133\%.$$

Here there is a total effect of +0.033%. If, however, the 40 ml. is exactly correct and the 10 ml. is 10 + 0.040, there is no standardisation correction, but the correction for ammonia absorbed is

$$\frac{-0.040}{(40 - 10.04)} \times 100 = -0.133\%.$$

The maximum error here is therefore expressed by

$$\frac{\text{Tolerance}}{\text{acid neutralised}} \times 100,$$

the tolerance and acid neutralised being expressed as burette volumes of alkali. Adding this tolerance error of 0.13 to 0.33, we get 0.51% as the total range.

In the above example it was supposed that 10 ml. or 25% of the acid equivalent were used in back titration. In the same way we may consider the total variable error for different percentage back titrations, and these are illustrated in Fig. 47.

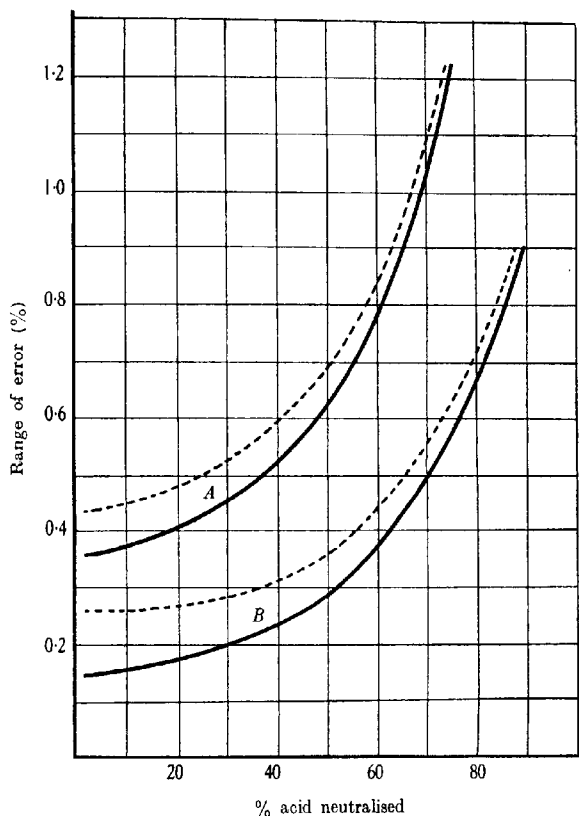


FIG. 47. Curves of volumetric error in the classical Kjeldahl determination.

The dotted lines are for pipetted fluid (3 ml.) and the smooth curves for weighed solid.

The upper two curves *A* give the total error range, including standardisation errors and burette tolerance of the 50 ml. burette. The lower two curves (*B*) give the full error range of the discrimination error as described in text.

It will be seen that a very marked effect on the error is produced as the back titration increases, and it is always advisable to keep it below the 25% level wherever possible.

Fig. 47 shows also (thick line) the error involved where a weighed solid is used for nitrogen analysis instead of a pipetted volume of 3 ml.

The curves give the *full range* of error, the upper curves including that for standardisation and bore tolerance (dotted lines) errors, the lower curves giving the discrimination error.

From the above treatment the impurity error in the standardising substance has been omitted. In the case of sodium oxalate as prepared above, and responding to the tests given, the maximum error is 0.04%, which may be treated as the limit of a variable error and added to the above range.

**The constant error allowances.** The method to be described reduces all the constant errors or allowances to a simple and easily workable form.

If  $X$  be the ml. of alkali solution from the burette corresponding to the ammonia analysed, then

$$X \times 0.05 \times 14 = \text{mg. ammonia } N \text{ absorbed} \\ (\text{using } 0.05N \text{ alkali})$$

and 
$$\frac{0.7X}{V} = Y,$$

where  $Y$  is the mg.  $N/\text{ml.}$  of fluid analysed, of which  $V$  ml. were pipetted for analysis. To allow for the constant errors we multiply above and below by the factors required to convert pipetted and buretted fluid from nominal to real equivalents at  $t^\circ$  (Table XXXIII), so that, in full

$$0.7X \left( 1 + \frac{B_{20} + S_{20} - \eta}{100} \right) = VY \left( 1 + \frac{P_{20} + S'_{20} - \eta}{100} \right),$$

where  $B_{20}$ ,  $P_{20}$ ,  $S_{20}$  and  $S'_{20}$  are the % factors for the burette, pipette, alkali, and solution for analysis, at  $20^\circ$ ,  $\eta$  being the special temperature factor as in Table XXXIII.

From the above equation (using the principle for small errors in Chapter XXIX), we have

$$\frac{0.7X}{V} \left( 1 + \frac{B_{20} - P_{20} + S_{20}}{100} \right) = Y \left( 1 + \frac{S'_{20}}{100} \right).$$

The required final factor  $S'_{20}$  is therefore given by

$$S'_{20} = B_{20} - P_{20} + S_{20}.$$

This may be expressed in a final form by expanding the standardising factor for the alkali  $S_{20}$ . This depends on whether it has been standardised against solid or using the preliminary acid titration.

From Table XXXIII, and inserting the expansion of  $S_{20}$  for each case, we get

$$S'_{20} = -D - P_{20} + \eta \quad (\text{solid standardisation})$$

$$\text{or} \quad S'_{20} = -D + S_{20}^{ac} + P_{20}^{ac} - P_{20}, \quad (\text{standardisation from preliminary acid titration}).$$

In the second equation  $S_{20}^{ac}$  and  $P_{20}^{ac}$  are the % constant errors of the acid strength and the 25 ml. pipette for delivering the acid.

If we neglected a standardisation of the two pipettes and assumed that they could vary in plus or minus value up to the maximum of their tolerance, then the two will add as 0.1% for the 25 ml. and 0.3% for the 3 ml. standard pipettes or a total of 0.4%. By the omission therefore of this easy standardisation from the five delivery mean, we double our variable error range, the 0.4% being added on to the limits of the variable error considered above.

**Constant error allowances using weighed solid for analysis.** The form of the allowance may be obtained as above. Here the value of the burette reading corresponding to the neutralised ammonia is multiplied by the factor for converting such burette deliveries from nominal to actual equivalent value. This factor is given in Table XXXIII as

$$(-\eta + B_{20} + S_{20}).$$



We may write therefore

$$F(\text{solid}) = -\eta + B_{20} + S_{20}.$$

On expanding  $S_{20}$  the alkali factor according as it has been standardised against solid or by the preliminary titration (from Table XXXIII), we get

$$F(\text{solid}) = -D \quad (\text{alkali standardised by solid})$$

$$\text{or} \quad = -D + S_{20}^{ac} + P_{20}^{ac} - \eta \quad (\text{alkali standardised by the preliminary acid titration}).$$

In Table XXXV the constant error allowances and the variable error ranges for given conditions are summarised.

TABLE XXXV

<i>Analysed substance.</i>	<i>Conditions of standardisation.</i>	<i>Discriminating range as back titration approaches zero. (Full duplicates for each sample.) (%)</i>	<i>Absolute error range of determination allowing for standardisation and burette tolerance. (%)</i>	<i>Factor for constant error (%).</i>
Weighed solid.	Alkali standardised by solid.	0.14	0.35	$-D$
Weighed solid.	Alkali standardised by preliminary titration.	0.14	0.36	$-D + S_{20}^{ac} + P_{20}^{ac} - \eta$
Pipetted fluid (3 ml.).	Alkali standardised by solid.	0.26	0.42	$-D - P_{20} + \eta$
Pipetted fluid (3 ml.).	Alkali standardised by preliminary titration.	0.27	0.43	$-D + S_{20}^{ac} + P_{20}^{ac} - P_{20}$

In the table it is considered that 20 ml. of acid are pipetted into the receiving flask by a 20 ml. pipette and that the alkali

used to titrate is half the acid strength. It is also taken that the burette is read to 0.2 of a final division.

Reading of this accuracy is very unusual, but if read to 0.5 of a small division (and there is no point in further inaccuracy), this will cause a change in the figure 0.22 above to 0.25% and the 0.37% to 0.40%. The symbols in the final column are described throughout the account in the text.

$P_{20}$  pipette factor for analysed fluid at 20°,

$P_{20}^{(ac)}$  " " " acid " 20°,

$S_{20}^{(ac)}$  acid factor for 20°,

$D = \frac{\text{titration fig.} - 40}{40} \times 100$ , where 40 ml. is the  
nominal expected  
value before stand-  
ardising.

$\eta$  is a temperature correction given in Table XXXIII.

**Analysis of the volumetric error of a micro Kjeldahl procedure using the micro diffusion technique.** Here we may consider the pipetting of 0.2 ml. fluid, such as urine, and its incineration, using 1 ml. of concentrated sulphuric acid, making up the incinerated mixture to mark with water in a 50 ml. volumetric flask and the removal of 0.5 ml. of this for analysis. 1 ml.  $N/200$  acid for the absorption and  $N/140$  alkali from the horizontal burette (or the solutions of Range I, Table V) are assumed.

In a consideration of the error involved only a procedure will be considered which is convenient and easily attained, and which at the same time may be expected to give the high accuracies stated. We shall suppose 3 simple tube pipettes constructed to deliver 0.2, 0.5 and 1.0 ml. respectively, and with fine tips as commented upon in Chapters V and XXIV. The length of each pipette is considered to be 20 cm. up to the mark and to take 20 seconds to deliver. As already shown (Chapter XXV), the coefficients of variation are then 0.065, 0.041 and 0.029 respectively.

With regard to the burette, on titrating we shall assume that an end-point emergence of one small division on the burette can

be made easily (though much less can in fact be controlled) and transferred by a fine pyrex rod or by direct contact, and that the burette reading is taken to half of one small division. This gives a manipulation error of 0.3 c.mm. (standard deviation).

In delivering from the burette it is supposed that the meniscus travels at 1 cm. per second, giving a delivery error of 0.065%. We may consider then the following :

Variable error of pipetting 0.2 ml. fluid	$= 0.065 \times 50\gamma N \times 10^{-2}$ .
Variable error of taking 0.5 ml. of the dilution	$= 0.041 \times 50\gamma N \times 10^{-2}$ .
Variable error of pipetting 1.0 ml. acid	$= 0.029 \times 70\gamma N \times 10^{-2}$ .
Variable error of titrating back 0.2 ml.	$= 0.163 \times 20\gamma N \times 10^{-2}$ .
(including delivery and manipulation errors)	

$$\text{whence } \sigma = 10^{-2} \sqrt{3 \cdot 25^2 + 2 \cdot 05^2 + 2 \cdot 03^2 + 3 \cdot 26^2} \gamma \text{ am. } N.$$

$$= 5 \cdot 4 \times 10^{-2}.$$

Amount analysed is  $50\gamma$  am.  $N$ , therefore the coefficient of variation is 0.108%.

Hence the total range is 0.324 and is the discriminating range using a duplicate determination, being therefore practically the same as in the full macro Kjeldahl.

If a duplicate analysis with each sample is carried out on the diluted fluid after incineration (and the Unit procedure is particularly suitable for this), we would get

$$\sigma = 10^{-2} \sqrt{3 \cdot 25^2 + 0 \cdot 5 \times 2 \cdot 05^2 + 0 \cdot 5 \times 2 \cdot 03^2 + 0 \cdot 5 \times 3 \cdot 26^2} \gamma$$

$$= 4 \cdot 5 \times 10^{-2} \gamma.$$

This gives with  $50\gamma$  ammonia  $N$  analysed a coefficient of variation of 0.090 and a range of 0.270 for the full duplicate of such a procedure.

This error range (using double Unit determinations of the diluted incinerated fluid) is therefore on the purely volumetric side, at least as accurate as the full macro Kjeldahl procedure, since from Fig. 47 it will be seen that for the analysis of 3.0 ml. pipetted fluid by the classical Kjeldahl procedure a discriminating error of 0.35% is obtained with a 20% back titration.

If we analyse, instead of pipetted fluid, a weighed sample by a micro balance so that the variable error of introducing

sample is practically eliminated, we get for a back titration of 28% :

$$\begin{aligned} v_t &= \sqrt{0.090^2 - 0.065^2} \\ &= 0.062, \end{aligned}$$

or a range of  $\pm 0.19$  which is closely similar to that with the macro procedure, which gives  $\pm 0.20$ .

*This comparison refers only to the volumetric procedure, but it is practically certain that the simple passive diffusion absorptions in the Unit will introduce less further error (in fact practically negligible) than the large scale high temperature distillations of the classical Kjeldahl procedure.*

**Absolute error range.** This will include the variable error of standardisation as before, and the bore tolerance of the burette if this is considered inconvenient to determine, though here it is much easier than with larger burettes and can be done by measuring a length of mercury at various positions. At the same time it may be noted that the use of high quality thermometer tubing practically eliminates the need of this allowance or investigation, and gives therefore one further advantage. The standardisation correction follows the previous account. There is a small further variable error included since the standardised acid of strength about  $0.1N$  will need to be diluted to  $N/200$ , but when this is done using a 10 ml. standard pipette, the extra error has no appreciable effect on the whole.

The constant error allowances may be also used as in Table XXXIII. The effect of the dilution on the  $S_{20}^{ac}$  factor of the  $\frac{M}{10}$  acid may be obtained from  $S'_{20}^{ac} = S_{20}^{ac} - F_{20} + P_{20}$ , where  $F_{20}$ ,  $P_{20}$  are the factors for pipette and flask and  $S_{20}^{ac}$  of the comparatively strong solution.

## APPENDIX

### CHAPTER XXXII

#### UREA EXCRETION AS RENAL FUNCTION TEST

THE excretion of urea as the most important nitrogenous end product in man has been much used as a test of renal function. It acts as a very useful measure of that part of the total renal functioning which is concerned with the excretion of nitrogenous waste products. Obviously it does not in itself give any index of the water or electrolyte regulation.

The urea excretion by the kidney may be easily shown to be influenced by the blood urea concentration, and the urine rate at constant body weight. Clearly therefore we must consider any renal output of urea with regard to the blood urea concentration and the urine volume.

*What we are seeking here is a comparison of the given urea excretion with the average normal figure.* This we can do in two ways, apparently different, but which are in fact identical. We could calculate what the excretion of the given subject would be at standard blood urea concentration and standard urine volume, allowing for the effect of the difference from the standard in these variables; and then compare such a calculated excretion with the average normal figure at such standard values.

On the other hand we could compare directly with the average normal excretion at the observed blood concentration and urine rate. The mean normal excretion so obtained may be written  $VC_u'$ , where  $V$  is the subject's urine volume in unit time and  $C_u'$  the average normal urine concentration at this volume and given blood concentration. The subject's actual excretion may be written  $VC_u$ . In comparing these we may obviously neglect  $V$ , which is the same in both, so that our requirement is the figure for the average normal urine concentration under the given conditions of blood concentration and urine rate. *Such a figure could be obtained most readily from*

tables compiled from a study of the available figures for excretion in the normal human subject.

TABLE XXXVI. Average normal urea concentration ratios for a body weight of 70 kilos (urine/blood)

Volume of urine ml. per hour.	0	1	2	3	4	5	6	7	8	9	Volume of urine ml. per hour.
20	96.2	93.6	91.2	88.9	86.8	85.0	83.3	81.8	80.3	78.9	20
30	77.6	76.3	75.1	73.9	72.7	71.6	70.6	69.5	68.5	67.5	30
40	66.5	65.6	64.8	64.0	63.1	62.3	61.6	60.9	60.2	59.4	40
50	58.8	58.1	57.4	56.8	56.2	55.7	55.1	54.5	53.9	53.3	50
60	52.8	52.3	51.8	51.3	49.8	50.4	50.0	49.6	49.1	48.7	60
70	48.3	47.9	47.5	47.1	46.7	46.3	46.0	45.6	45.2	44.8	70
80	44.4	44.1	43.8	43.5	43.2	42.9	42.6	42.3	42.0	41.7	80
90	41.4	41.1	40.8	40.6	40.3	40.0	39.8	39.5	39.3	39.0	90
100	38.8	38.5	38.2	38.0	37.8	37.6	37.3	37.1	36.9	36.7	100
110	36.5	36.3	36.1	35.9	35.7	35.5	35.3	35.1	35.0	34.8	110
120	34.6	34.4	34.2	34.0	33.8	33.6	33.4	33.3	33.1	32.9	120
130	32.7	32.5	32.3	32.2	32.0	31.8	31.6	31.5	31.3	31.2	130
140	31.1	30.9	30.7	30.6	30.4	30.3	30.2	30.0	29.8	29.7	140
150	29.6	29.5	29.3	29.2	29.1	28.9	28.8	28.7	28.6	28.4	150
160	28.4	28.3	28.2	28.0	27.9	27.8	27.6	27.5	27.4	27.3	160
170	27.1	27.0	26.8	26.7	26.6	26.5	26.4	26.3	26.1	26.0	170
180	25.9	25.8	25.7	25.6	25.5	25.4	25.3	25.2	25.1	25.0	180
190	25.0	24.9	24.8	24.7	24.5	24.4	24.3	24.2	24.1	24.0	190
	00	10	20	30	40	50	60	70	80	90	
200	23.9	23.0	22.2	21.4	20.7	20.0	19.3	18.7	18.1	17.6	200
300	17.1	16.6	16.1	15.6	15.2	14.8	14.4	14.0	13.7	13.3	300
400	13.0	12.6	12.3	12.0	11.7	11.4	11.1	10.8	10.6	10.4	400
500	10.2	9.98	9.76	9.54	9.32	9.10	8.90	8.72	8.53	8.35	500
600	8.19	8.04	7.90	7.75	7.60	7.45	7.30	7.15	7.00	6.85	600
700	6.72	6.60	6.49	6.38	6.26	6.14	6.03	5.92	5.81	5.71	700

Such tables though convenient would be lengthy. However, a much shorter table, and nearly as convenient (Table XXXVI), may be used, giving the average number of times the blood urea is concentrated in the urine of the normal subject. This multiplied by the blood concentration gives the required urea concentration in the urine, and dividing this figure into the actual concentration for the subject and multiplying by 100 gives the urea excretory function expressed as a mean 'normality ratio'. The use of Table XXXVI in this way

depends on the fact—upon which all are agreed—that over the clinical range the urea excretion at constant urine rate is proportional to the blood concentration.

The 'normality ratio' as a direct and permanent conception has as a theoretical counterpart here the urea 'pressure', or the diffusion pressure of the urea in the tubule lumen which with a urine rate slowed to zero would be in equilibrium with the urea in the cells and prevent any further emergence of urea therefrom. An equation based on such a conception expresses the actual data much better than other equations hitherto proposed (*vide* Table XXXVII).

*The urea normality ratio for a given subject may be formally defined then as his urea excretion (or urine concentration) expressed as a percentage of the average normal value at the same blood urea and urine rate.*

Equations or formulae need have no connection with the determination of such a figure, which we could suppose obtained with the aid of normal excretion tables. It will be shown subsequently that for the purpose of determining renal function all the equation does, even when ideally exact, is to provide us with a means whereby the average normal figure may be obtained without tables, or alternatively to assist in their construction.

TABLE XXXVII

Range of $\bar{V}$ ml./hr.	Mean $\bar{V}$ ml./hr.	N (No. of sets).	$\frac{VC_u}{C_b}$	General pressure formula (mean values).	Clearance formulae (mean values).	
					II.	I.
0-20	15.9	8	13.6	78.8	81.5	
20-40	31.2	67	23.6	99.4	101.1	
40-60	48.5	102	29.4	101.1	100.9	
60-80	68.1	77	33.7	100.7	97.6	
80-100	87.5	40	36.7	99.1	93.8	
100-120	108	19	39.9	99.8	91.6	
120-160	139	36	45.9	105.0		101.8
160-200	180	15	46.7	100.2		103.4
200-300	235	22	47.5	96.1		105.1
300-400	375	16	51.7	99.3		113.5
400-500	448	34	49.8	96.1		110.4
500-600	551	30	50.5	100.7		112.0
600-700	640	26	46.5	95.5		103.1
> 700	753	15	48.5	105.6		107.1

Sources of the data :

	<i>Sets.</i>	<i>Subjects.</i>
Walker and Rowe (93) - - - -	52	15
Addis and Watanabe (86) - - - -	106	33
McLean (95) - - - -	107	34
Austin <i>et al.</i> (94) - - - -	32	2
Conway and Dillon (160) - - - -	77	77
Addis and Drury (161) - - - -	72	4
Conway and Donovan (unpublished data) - -	22	2
Pollard (162) - - - -	39	1
	507	168

General diffusion pressure formula

$$\frac{\sqrt{V}(C_u - C_b)}{441C_b e^{-\alpha}} = 100 \text{ (mean)}$$

'clearance' equations,

$$\frac{\sqrt{V}C_u}{4 \cdot 18C_b} = 100 \text{ (mean)}; \quad \frac{VC_u}{45 \cdot 1C_b} = 100 \text{ (mean)}.$$

The full statistical decision between the 'clearance' formulae, the modification by Dominguez (163), and the 'pressure' formula will be considered elsewhere.

The comparison of the general 'pressure' equation and the so-called 'clearance' equations in predicting the output of urea with change of urine volume at constant blood concentration is given in Table XXXVII and Fig. 48. The use of the equations alone—without tables—will be considered in a subsequent section, though it may be noted again that tables are ideally the most exact, when constructed from the actual data of the normal excretion, and involve no theoretical interpretation in the ordinary sense.

#### The determination of the urea normality ratio.

*The collection of the blood and urine samples.* The test may be carried out at any time of the day and without intake of urea or other substance, though a glass of water prior to the test is advisable, but not essential; also since meals have the effect of raising the excretory power of the kidney within the first few hours after a meal, it would seem advisable to carry out these tests in the hour preceding a meal.

The bladder is firstly emptied, the exact time being noted and the urine discarded. After half an hour a sample of blood is taken from the finger in the manner described for the blood



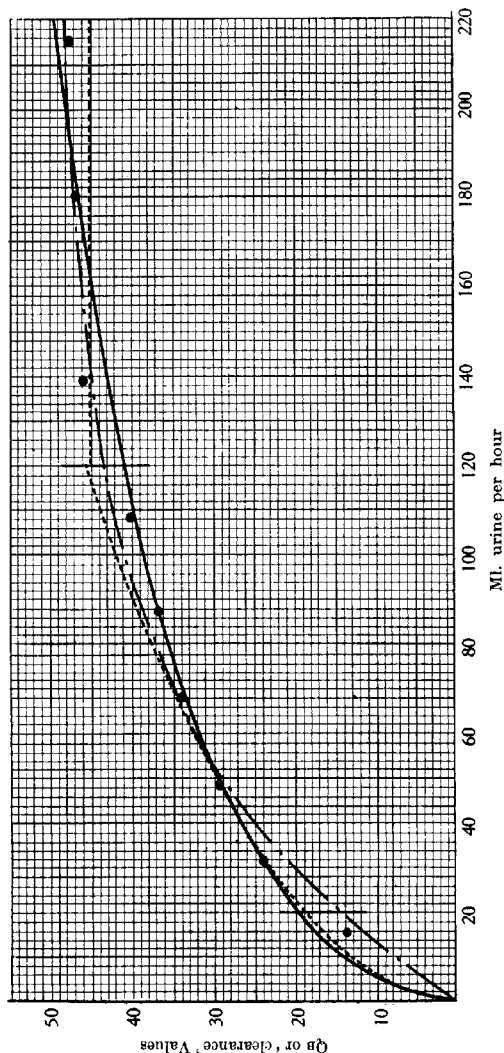


FIG. 48. The dots give the means of the excretion per unit blood concentration at varying urine rates for the normal human subject.

Line — curves from general diffusion-pressure equation.

" - - - - - from 'clearance' equations.

" ..... from the equation of Dominguez.

The best numerical values—statistically—were used for the 'clearance' curves and for that of Dominguez (163). Between the two upright lines from 20 ml./hr. to 120, there are 305 sets of observations from the general literature summarised. The means are almost exactly described by the diffusion-pressure equation. The comparative success of the Dominguez exponential equation (as considered by Dominguez) is very largely due to the fortuitous correspondence of the few observations below 20 ml./hr. His statistical treatment artificially exaggerates this correspondence. Clearly his curve—at its best (as here drawn)—is not the true or best descriptive curve, and it is obvious also from this and Table XXXVII that the diffusion-pressure curve is superior to the two clearance curves combined.

Since the dimensional values of the constant in the general diffusion pressure equation are altogether different from a mere volume, this reduces the 'clearance' concept to a dubious simplification, apart altogether from the fact that the accepted interpretation ends in a contradiction as shown elsewhere (ref. 186).

urea determination, and after one hour the bladder is emptied again and the volume in ml. or c.c. measured in a graduated cylinder. The weight of the subject is also noted.

**In the dilution of the urine for analysis a procedure may be adopted which is very convenient both for this purpose and the subsequent calculation of renal function.** The volume of urine per hour (corrected if necessary for change of body weight from a standard 70 kilos, according to the very simple formula below, though from 60–80 kilos body weight very little is gained by such corrections) is noted and the average normal concentration ratio read off from Table XXXVI, the urine being diluted this number of times. Thus if the average normal concentration ratio is found to be 76.3 (urine volume of 31 ml./hr.), 1 ml. urine is pipetted into a 200 ml. Erlenmeyer flask and 75.3 ml. water added from a burette. The water and urine are well and carefully mixed. This urine should then have exactly the same concentration as the blood if the subject's excretion corresponded to the average normal value. *The figure for the urea excretory function or the normality ratio is given at once on dividing the concentration of the blood urea into that of this diluted urine and multiplying by 100, the procedure being always the same, excepting the different figures for the necessary dilution required.* This calculation involves as a rule only one division (pipette and alkali factors cancel out) and is in principle the most accurate form in which the urea excretory test can be applied.

If tables are not available, the calculation from equations is given below.

**Correction of the urine volume for body weight, or surface.** This correction should be made on a surface relation since the urine volume goes proportionally thereto. The following simple formula gives a suitable correction factor.

$$\frac{W + 30}{100} = F. \quad (1a)$$

The value of  $F$ , which can be instantly written down, is divided into the urine volume obtained. The result gives a  $W^{\frac{2}{3}}$  correction to a standard body weight of 70 kilos over the range of

40 to 90 kilos, to within 2% of the exactly calculated value at the extremes of this range (amounting usually to less than one per cent. in the final ratio or deviation). A more exact correction than this for surface is unnecessary in the adult subject since the correlation between the mean urine volume and body weight is very low for the normal range of adult body weight and a quite negligible increase in accuracy is introduced with any such correction from 60 to 80 kilos.

A corresponding formula for the range of 12 to 35 kilos may be given as

$$\frac{W+10}{70} = F. \quad (2a)$$

This has the same accuracy as the first, showing at the extremes the same divergence from an exact  $W^{\frac{2}{3}}$  correction.

**Examples of the determination of urea normality ratios ( $R_u$ ).**

*Subject 1.* Body weight - - - 70 kilos.  
 Urine volume - - - 180 ml./hour.  
 Blood collected midway.

From Table XXXVI at 180 ml./hr. the average normal concentration ratio is 25.9, so that to 1 ml. urine 24.9 ml. water were added.

Diluted urine concentration - - 32.3 mg./100 ml.  
 Blood concentration - - - 28.5 mg./100 ml.

$$\text{Normality ratio } (R_u) = \frac{32.3}{28.5} \times 100 = 113.$$

The tables are made out for a body weight of 70 kilos, so that no correction for weight occurs here.

If the urine were not diluted as above, and the concentration returned finally as 0.837%, the normality ratio is obtained by multiplying the blood concentration by 25.9 from Table XXXVI (corresponding to 180 ml./hr.) and dividing into  $0.837 \times 100$ , thus obtaining as before 113.

*Subject 2.* Body weight - - - 86 kilos.  
 Urine volume - - - 62.5 ml. per hour.  
 Blood collected midway.

From the weight formula above, we may write down immediately 1.16 as the divisor into 62.5 ml. and obtain 54 ml. as the corrected volume. From Table XXXVI this corresponds to a concentration ratio of 56.2, 55.2 ml. water being added to 1 ml. urine.

Diluted urine concentration - - 26.3 mg./100 ml.  
 Blood concentration - - - 46.7 mg./100 ml.

$$\text{Normality ratio } (R_u) = \frac{26.3}{46.7} \times 100 = 56.$$

*Subject 3.* Body weight . . . 64 kilos.  
 Urine volume . . . 14.0 ml./hr.  
 Blood collected midway.

Here no correction need be made for body weight as it lies in the region 60–80 kilos, though some may prefer to make it as a routine.

Making the correction will alter the volume to 14.9 ml./hr.

Table XXXVI begins at 20 ml./hr. since there is no apparent increase in urine concentration below this level, though more data are here desirable. Consequently the urine is regarded as being 20 ml./hr. and a dilution of 96.2 made; 95.2 ml. water being added to 1 ml. urine.

Diluted urine concentration . . . 21.4 mg./100 ml.

Blood concentration . . . 30.0 mg./100 ml.

Normality ratio ( $R_u$ ) =  $\frac{21.4}{30.0} \times 100 = 71$ .

#### Normal limits and distribution of the normality ratio ( $R_u$ ).

Fig. 49I gives the general distribution of the normal ratios or  $R_u$  values, and also the distribution for a single individual (histogram III, in which the dotted line gives the effect of a meal). The chief interest for the pathologist or clinician in such assessment of function lies in the consideration of a lower normal limit (already considered).

With regard to the normal limits, 5% of the  $R_u$  values fall below 70. These include no doubt a certain percentage of experimental errors. From 60 to 70 may be taken as a region of suspected subnormal functioning, to be certified by further determinations.

With regard to variability of  $R_u$  it may be said that even for the single individual there occur very appreciable variations in the renal functional power. When the observations are scattered throughout the day, and up to 120 ml. per hour, the standard deviation is 12.3 (probable error = 8.3). Nearly all the observations will then lie between  $\pm 25\%$  of the mean. When the observations are carried out each day at the same time the standard deviation is 9.5 (probable error 6.4). The difference is largely due to the effect of meals—but differences even in the normal range of body temperature appear also to have appreciable influence on the excretion. Distributions of  $R_u$  values are given in Fig. 24.

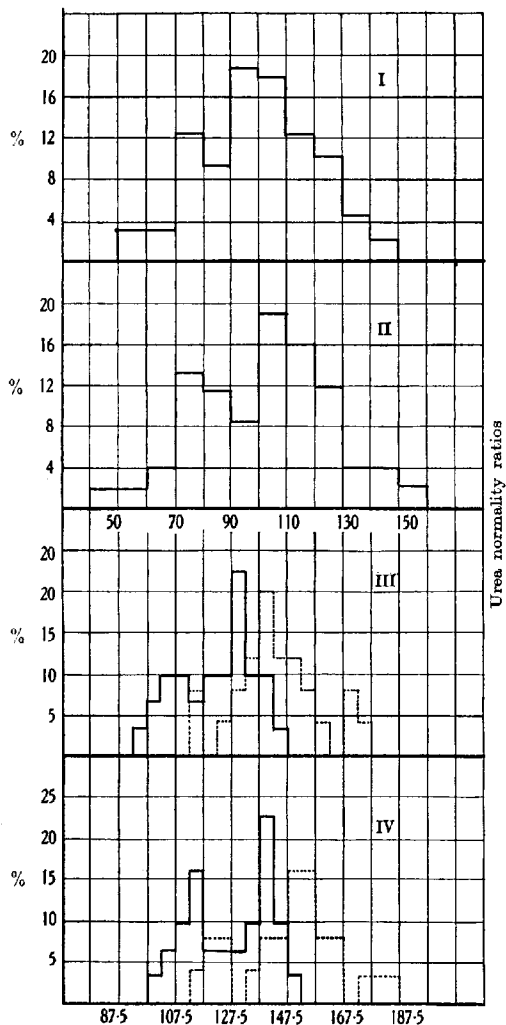


Fig. 49. Distribution of normality ratios for normal human subject.

- I. 169 obs.—data from general literature. Urine rate limited to 120 ml./hr. to show peaked distribution best.
- II. The same obs. in which the ratios were calculated from the first pressure equation (equation 7a in text). This corresponds to a 'clearance' distribution.
- III. 56 obs. on a subject before and after a light lunch (dotted line—after lunch with urine collection up to 90 mins.). Urine rate limited to 120 ml./hr.
- IV. The same obs. as in III, using the first pressure equation, corresponding to a 'clearance' distribution.

The use of equations instead of tables for renal function tests.

(1) *Demonstration that the value of the equation for this purpose lies in providing a figure for the average normal urine concentration under the given conditions.* Any urea excretion formula which may be considered as at all significant, and the mean normal value of which is represented as 100, can be written in the form

$$VC_u = 100 \times f(C_b, V, W), \quad (3a)$$

where  $VC_u$  is the excretion in unit time, and  $f(C_b, V, W)$  some function of the urine volume ( $V$ ), the blood concentration ( $C_b$ ) and the body weight ( $W$ ).

If on the right we insert the values for a given individual during an observation, we shall get nearly always values different from  $VC_u$  the actual excretion rate. However, the value of the expression  $\Sigma(VC_u' - VC_u)^2$  is (or should be) a minimum. Here  $C_u$  is the actual urine concentration and  $C_u'$  the calculated value;  $VC_u'$  being the value of the expression on the right of the previous equation. The value of the above expression can only be a minimum when  $VC_u'$  is the average normal figure under the given conditions (of blood concentration, etc.). Equation 3a may now be written

$$\frac{VC_u}{f(V, C_b, W)} = R_u (= 100 \text{ as the mean value}),$$

or, as we have seen

$$100 \times \frac{VC_u}{VC_u'} = R_u (= 100, \text{ mean value}), \quad (4a)$$

where  $VC_u'$  is the average normal excretion at given values of  $C_b$ ,  $V$  and  $W$ .

It is obvious that the equation—for the purpose in question—only provides us with a figure for the average normal output.

The theoretical significance of the equation whereby  $VC_u'$  is obtained is a second stage in the interpretation.

**Urea excretory equations.** It may be said that an equation is here valid just in so far as it predicts the output. The comparative accuracy with which it does this can be decided only by standard statistical procedure.

(a) *The general diffusion-pressure equation.* This for constant body weight may be written

$$V(C_u - C_b) = 440C_b V^{0.5} e^{-0.00152V}, \quad (5a)$$

which can be put in the form

$$\frac{C_u}{C_b} = 1 + 440 V^{-0.5} e^{-0.00152V}. \quad (6a)$$

The comparison of the prediction of the average excretion from equation 24 and the two 'clearance' equations is made in Table XXXVII, and in Fig. 48 (which latter also includes the Dominguez curve).

At low values of  $V$  this approximates to

$$\frac{C_u}{C_b} = 400 V^{-0.5} = \frac{400}{\sqrt{V}}.$$

At high values of  $V$ ,  $e^{-0.00152V}$  goes proportionally to  $V^{-0.5}$  over a wide range of urine volume (a mean deviation of about 5% from 200–800 ml. per hour), so that

$$\frac{C_u}{C_b} \text{ approximates to } V^{-0.5} \times V^{-0.5} \times \text{a constant} = \frac{\text{a constant}}{V}.$$

These approximate equations are again referred to below.

In the general pressure equation 6a, the expression on the right may be put in tabular form with changes of  $V$ . This table would give the mean concentration ratio of urine to blood at the given urine rates, and the result will be the same as when using Table XXXVI.

The significance of the urea diffusion pressure is that it represents the outward diffusing pressure of the urea from the tubules, which is balanced in a stationary urine by a high concentration in the tubule lumen and in which case no further urea is then excreted. When the urine begins to flow there is not time for the back diffusion pressure to equal the outer, so that urea is continuously excreted, and in accordance with the general diffusion pressure equation theoretically derived.

The general diffusion pressure equation is superior to both 'clearance' equations even over the special regions to which these latter apply.

\* For inulin there is a different coefficient of  $V$  in  $e^{-0.00152V}$ , and this equation holds for a much lower range of  $V$ .

(b) *The first 'pressure' equation.* Where tables are not available the next two equations give approximations, and are in their present numerical form somewhat more accurate than the corresponding 'clearance' formulae. The first is

$$\frac{\sqrt{V}(C_u - C_b)}{4C_b} \quad \text{or} \quad \frac{\sqrt{V}C_u}{4C_b} - R_{U'} = 100. \quad (7a)$$

The  $C_b$  may be taken as negligible compared with  $C_u$  so that the second form of the expression may be used instead of the first. This equation may be applied to a volume limit of 150 ml. per hour, up to which point it agrees to within 5% with the general diffusion pressure equation. As with the general diffusion pressure equation, the urine rate of 20 ml. per hour is used for all rates below this level. The first pressure equation gives values 5.7% too high at 20 ml. per hour and too low at 120 ml. per hour by 4.5%.

(c) *The second 'pressure' equation.* This is written

$$\frac{V(C_u - C_b)}{44C_b} = R_u'' = 100 \text{ (average normal value)} \quad (8a)$$

and applies beyond 150 ml. per hour.

At the highest urine volumes attainable, namely beyond 600 ml. per hour, it is to be expected theoretically that this equation will give slightly better figures than even the general diffusion pressure equation and, of course, better than the corresponding 'clearance' formula, (for other references to these equations and related ideas, see 167-174).

(d) *The third pressure equation.* (Of use only with the experimental animal at very high blood concentrations.)

This may be written

$$\sqrt{V}(C_u - C_b) = P_3 = 1.40 \text{ (for the rabbit)}. \quad (9a)$$

Here the value of  $P_3$  is not numerically adjusted to 100,  $V$  being expressed as ml. per minute and  $C_u$  and  $C_b$  as g./100 ml. *For the range of blood concentration beyond 200 mg./100 ml. the 'clearance' formulae altogether fail, the values altering upwards of five times or more with changing  $C_b$  and  $V$  when the  $P_3$  figure remains unchanged.*



Comparison of the 'clearance' formulae and the first and second 'pressure' equations. The 'clearance' formula (175, 176), corresponding to the first pressure equation, may be written

$$\frac{\sqrt{V}C_u}{4.18C_b} = \text{'clearance' as percentage of average normal value. (10a)}$$

The introduction of the volume as a square root function (94) was the novel feature of this equation and was introduced as follows: 'We have tried curves representing other functions of  $V$ , such as  $V^{\frac{1}{3}}$  and  $V^{\frac{2}{3}}$ , and  $\log V$ , without finding one that so nearly approximates the experimental results'.

The exact verification (within the sampling error) of the index of  $V$  in equation 10a as 0.50 was given by the present writer (165). The above 'clearance' formula gives values too low at 120 ml. per hour by 9.8%, the similar errors of the first 'pressure' equation being 4.5%, whereas the general 'pressure' equation shows no error here. The 'clearance' formula corresponding to the second 'pressure' equation and used beyond a urine rate of 120 ml. per hour may be written

$$\frac{VC_u}{45.1C_b} = \text{'clearance' (as a percentage of the average normal value). (11a)}$$

This 'clearance' equation (in similar form) was introduced by Marshall and Davis (177) and further developed by Pepper and Austin (178), Addis and Watanabe (86), Addis, Barnett and Shevky (179), Austin, Stillman and Van Slyke (94) showing definitely that a high rate was essential for its appearance.

The earliest attempts to express the urea excretion in an equational form, and taking account of the variables  $C_u$ ,  $C_b$  and  $W$ , were made by Ambard (180, 181). His equations may be regarded as having now only historical value.

The peaks in the distribution curve of normal renal function values. The distributions in Fig. 49 show these peculiarities. The first histogram is for  $R_u$  using Table XXXVI (limiting the value to 120 ml./hr.), the second for  $R_u'$  (1st 'pressure' equation or 'clearance' equation to 120 ml./hr. The  $R_u'$  values with the 'clearance' equation are slightly lower). It will be seen

that for both distributions there is a marked grouping around two main peaks. The grouping here into peaks has a different significance than for the previous distribution of blood concentration, etc. The latter could, for example, be varied almost at will by increasing the protein and water intake. It is intimately dependent on these factors as well as on the renal power to excrete urea. In the renal function values, however, the main apparent factors have been allowed for, so that variability here tending to two modes has an ultimate metabolic significance for the kidney. The effect of meals arises for consideration. In the first to the second hour there is in fact a rather marked rise in the urea excretory power, as shown in histograms III and IV of  $R_u$  and  $R_u'$  values for the single individual. The dotted lines represent the effect of a light lunch, the mid-point of the urine collection being in the first to the second hour after the meal. The thick line represents the distribution of 31 values in the third to the fourth hour after breakfast and immediately prior to lunch, and this distribution shows a marked grouping into two regions of activity.

Each of these 31 values was obtained on a separate day, (the conditions being strictly normal and routine), and the mid-time of the urine collection was always in the third to the fourth hour after breakfast.

Similar peaking appears in the general distribution of values in histograms 1 and 2. Such data show a marked grouping into two peaks which can have no relation to the metabolic effect of meals. (A similar distribution was also observed for functional values before breakfast, ref. 173).

We must assign these groupings to obscure metabolic causes. It is of interest to note, however, that similar phases in the general metabolic curve have been observed by O'Connor (182-184), and a very striking resemblance in slope and phasic formation has been noted in the curve of urea secretory function for the experimental animal and that of the general metabolic curve when plotted against body temperature (173).

Comparing the histograms for the single individual and for the general group, it would seem that in the general histogram

a third peak illustrating the effect of meals should be evident with a sufficiently large number of data. This peak would lie some distance to the right of the second peak, and is, in fact, already manifest to some extent.

The single individual from whom the data for histograms were obtained had an exceptionally high mean value for the urea excretory function. For comparative purposes the histograms are so arranged that the first peak in the distribution for this individual coincides with the first peak in the general distribution.

With regard to the theory underlying the general diffusion-pressure equation, considerations of space forbid any detailed presentation here. It may be noted that it involves the active excretion of urea and of other such typical urinary substances by the mammal. The active concentration of urea by the isolated kidney of the frog (*i.e.* by the renal cells in complete absence of a glomerular fluid) was first demonstrated here (Conway and Kane, 171). This has been ignored by some writers (*e.g.* Smith, 185) in recent accounts, and the author takes the opportunity of re-affirming it.

Readers accustomed to the usual presentations of 'clearance-reabsorption' views will find the superiority of the general diffusion-pressure equation somewhat surprising, but possibly not significant. The following points may therefore lead to a wider understanding and discussion.

- (1) It is assumed that inulin clearances measure the volume of the glomerular filtrate, all the inulin passing through the glomeruli, and none being reabsorbed. This idea leads to the contradiction that urea can then be shown to be reabsorbed in proportion to its lumen concentration, and again that the absorption of urea is independent of this factor. This contradiction has been pointed out in *Nature* (186), and so far remains unanswered.
- (2) It is supposed that the cells of the mammalian kidney are impermeable to inulin. The fact is that inulin can permeate renal sections under the most suitable conditions up to 30-50% of the total water content, whereas it permeates muscle to not more than 8%. Similarly, freshly excised kidneys perfused with cyanide, etc., show an inulin permeation of 50% (187).
- (3) It is likewise believed that only the most improbable assumptions can explain how inulin, creatinine and cane sugar, etc., give almost identical 'clearances', whereas such agreements arise naturally out of a diffusion-pressure theory. Granting that the kidney is working at its highest efficiency, then the equilibrium

concentration of a substance (passing freely through the cells) will tend to go inversely as the diffusion coefficient. This is so, because the work required to prevent the back diffusion will go in inverse relation to this figure. On the other hand, the amount of substance passing out into the stream of glomerular fluid will go directly as the diffusion coefficient, so that this value tends to cancel out in the final result. It is scarcely surprising, therefore, that many substances will show great similarity of excretion under certain conditions.

- (4) There is a very large back reabsorption of water required in these 'clearance-reabsorption' views, and such reabsorption is quite unsupported by the Höber dye experiments, or by the cyanide perfusion experiments of Starling and of later workers, or again by the effects of cooling, etc. Such opposing evidence is not even considered in Smith's account (185).
- (5) Lastly, the following very curious implications of the theory of the glomerular filtrate coinciding with the inulin 'clearance' may be pointed out.

It would appear to be now rather generally believed that the frog's kidney actively excretes urea. This power of direct excretion of urinary substances is regarded as lost by the mammalian kidney, which must filter off enormous amounts of water and reabsorb it. At the same time the mammalian kidney must—it is considered—be able to excrete actively many complex substances such as diodrast, phenol red, etc., which never entered it until injected by the physiologist. These substances are regarded as being excreted *in mistake* for something else. While excreting therefore these strange molecules *by mistake*, but with great efficiency, it appears that the renal cells have *lost* their power to excrete the ordinary constituents of the urine. An interpretation containing less aberrant anthropomorphism has been alluded to by Smith (185)—with unexpected humour—as 'against all the facts'.

Considerations of space forbid any further criticism.

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